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P/1259-438

MEDICAL USES OF A SELECTIVE ESTROGEN RECEPTOR
MODULATOR IN COMBINATION WITH SEX STEROID
PRECURSORS

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FIELD OF THE INVENTION

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The present invention relates to a method for treating or reducing the likelihood of acquiring osteoporosis, hypercholesterolemia, hyperlipidemia or atherosclerosis using a novel combination therapy on susceptible warm-blooded animals, including humans. In particular, the combination includes administering a selective estrogen receptor modulator (SERM) and raising the patient's level of precursor to sex steroids, said precursor being selected from the group consisting of dehydroepiandrosterone (DHEA), dehydroepiandrosterone sulfate (DHEA-S), and androst-5-ene-3 β ,17 β -diol (5-diol). The invention also relates to kits and pharmaceutical composition for practicing the foregoing combination.

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BACKGROUND OF THE RELATED ART

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Man is thus unique, with some other primates, in having adrenals that secrete large amounts of the precursor steroids dehydroepiandrosterone sulfate (DHEA-S) and dehydroepiandrosterone (DHEA) which are converted into androstenedione (4-dione) and then into active androgens and/or estrogens in peripheral tissues (Labrie et al., In: Important Advances in Oncology. Edited by V.T. de Vita, S. Hellman, S.A. Rosenberg. J.B. Lippincott, Philadelphia, 193-217, 1985; Labrie, Mol. Cell. Endocrinol., 78: C113-C118, 1991; Labrie, et al., In Signal Transduction in Testicular Cells. Ernst Schering Research Foundation Workshop. Edited by V. Hansson, F.O. Levy, K. Taskén. Springer-Verlag, Berlin-New York (Suppl. 2), pp. 185-218, 1996; Labrie et al., Steroids, 62: 148-158, 1997). In a recent study (Labrie, et al., J. Clin. Endocrinol. Metab., 82: 2403-2409, 1997), we have described a dramatic decline in the circulating levels of dehydroepiandrosterone (DHEA), DHEA-sulfate (DHEA-S), androst-5-ene-3 β ,17 β -diol (5-diol), 5-diol-S, 5-diol fatty acid esters, and androstenedione in both men and women between the ages of 20 and 80 years.

Despite the marked fall in endogenous androgens in women during aging, the use of androgens in post-menopausal women has been limited mainly because of the fear of an increased risk of cardiovascular disease as based upon older studies showing an unfavorable lipid profile with androgens. Recent studies, however, have shown no significant effect of combined estrogen and androgen therapy on the serum levels of cholesterol, triglycerides, HDL, LDL, and HDL/LDL ratio when compared to estrogen alone (Sherwin et al., Am. J. Obstet. Gynecol., 156:

5 414-419, 1987). In agreement with these observations, we have shown
that DHEA, a compound having a predominantly androgenic influence,
has apparently no deleterious effect on the serum lipid profile (Diamond,
et al., J. Endocrinol., 150: S43-S50, 1996). Similarly, no change in the
10 concentrations of cholesterol, its subfractions or triglycerides, over a
treatment with estradiol alone has been observed after 6 months of
therapy with estradiol + testosterone implants (Burger et al., Br Med. J.
Clin. Res. Ed., 294: 936-937, 1987). It should be mentioned that a study in
man has shown an inverse correlation between serum DHEA-S and low
15 density lipoproteins (Parker et al., Science, 208: 512-514, 1980). More
recently, a correlation has been found between low serum testosterone
and DHEA and increased visceral fat, a parameter of higher
cardiovascular risk (Tchernof et al., Metabolism, 44: 513-519, 1995).

Five-diol is a compound biosynthesized from DHEA through the action
20 of reductive 17 β -hydroxysteroid dehydrogenase (17 β -HSD) and is a weak
estrogen. It has an 85-fold lower affinity than 17 β -estradiol (E₂) for the
estrogen receptor in rat anterior pituitary gland cytosol (Simard and
Labrie, J. Steroid Biochem., 26: 539-546, 1987), further confirming the data
obtained on the same parameter in human myometrial and breast cancer
25 tissue (Kreitmann and Bayard, J. Steroid Biochem., 11: 1589-1595, 1979;
Adams et al., Cancer Res., 41: 4720-4926, 1981; Poulin and Labrie, Cancer
Res., 46: 4933-4937, 1986). However, at concentrations well within the
range of the plasma levels found in adult women, 5-diol enhances cell
proliferation and progesterone receptor levels in human mammary
30 tumor ZR-75-1 cells (Poulin and Labrie, Cancer Res., 46: 4933-4937, 1986),
and increases the estrogen-dependent synthesis of the 52 kDa

5 glycoprotein in MCF-7 cells (Adams et al., Cancer Res., 41: 4720-4926,
*1981).

10 As mentioned above, it is known that the serum levels of DHEA,
DHEA-S and 5-diol decrease with age and correspondingly, that there is
a dramatic age-dependent reduction in the formation of androgens and
estrogens in peripheral target tissues. Such changes in DHEA-S and
DHEA secretion result in a marked decrease in the biochemical and
cellular functions stimulated by sex steroids. As a result, DHEA and
DHEA-S have recently been used in the treatment of a variety of
15 conditions which are associated with decrease and/or imbalance in the
levels of sex steroids.

Osteoporosis, a condition which affects both men and women, is
associated with a decrease in androgens and estrogens. Estrogens have
20 been shown to decrease the rate of bone degradation while androgens
have been shown to build bone mass. However, estrogen replacement
therapy commonly used against osteoporosis requires the addition of
progestins to counteract the endometrial proliferation and the risk of
endometrial cancer induced by estrogens. Moreover, since both
25 estrogens and progestins are thought to increase the risk of breast cancer
(Bardon et al., J. Clin. Endocrinol. Metab., 60: 692-697, 1985; Colditz et al.,
N. Engl. J. Med., 332: 1589-1593, 1995), the use of estrogen-progestin
replacement therapy is accepted by a limited number of women and,
usually, for too short periods of time.

30 Several studies suggest that osteoporosis is a clinical manifestation of
androgen deficiency in men (Baran et al., Calcif. Tissue Res. 26: 103-106,

1978; Odell and Swerdloff, West J. Med. 124: 446-475, 1976; Smith and Walker, Calif. Tissue Res. 22 (Suppl.): 225-228, 1976).⁴ Androgen therapy, as observed with nandrolone decanoate, has been found to increase vertebral bone mineral density in postmenopausal women (Need et al., Arch. Intern. Med., 149: 57-60, 1989).⁵ Therapy of postmenopausal women with nandrolone increased cortical bone mineral content (Need et al., Clin. Orthop. 225: 273-278, 1987). Androgenic side-effects, however, were recorded in 50% of patients. Such data are of interest since while almost all present therapies are limited to a reduction of bone loss, an increase in bone mass was found with the use of the anabolic steroid nandrolone. A similar stimulation of bone formation by androgens has been suggested in a hypogonadal male (Baran et al., Calcif. Tissue Res. 26: 103, 1978). A stimulation of bone formation in postmenopausal women treated with DHEA for 12 months is reported in Labrie et al. (J. Clin. Endocrinol. 82: 3498-3505, 1997).

DHEA (450 mg/kg, b.w., 3 times a week) markedly delayed the appearance of breast tumors in C3H mice which were genetically bred to develop breast cancer (Schwartz, Cancer Res. 39: 1129-1132, 1979). Moreover, the risk of developing bladder cancer was found to be increased in men having lower serum DHEA levels (Gordon et al., Cancer Res. 51: 1366-1369, 1991).

U.S. Patent Application U.S. 5,550,107 relates to a method of treatment of breast and endometrial cancer in susceptible warm-blooded animals which may include inhibition of ovarian hormonal secretion by surgical means (ovariectomy) or chemical means (use of an LHRH agonist, e.g. [D-Trp⁶, des-Gly-NH₂¹⁰]LHRH ethylamide, or antagonist) as part of a

5 combination therapy. Antiestrogens, androgens, progestins, inhibitors of
sex steroid formation (especially of 17β -hydroxysteroid dehydrogenase-
or aromatase-catalyzed production of sex steroids), inhibitors of prolactin
secretion and of growth hormone secretion and ACTH secretion are
discussed. A counterpart thereof has been published under international
10 publication number WO 90/10462.

In addition, cardiovascular diseases have been associated with decreased
serum levels of DHEA and DHEA-S and both DHEA and DHEA-S have
been suggested to prevent or treat these conditions (Barrett-Connor et al.,
15 N. Engl. J. Med. 315: 1519-1524, 1986).

In aged Sprague-Dawley rats, Schwartz (in Kent, Geriatrics 37: 157-160,
1982) has observed that body weight was reduced from 600 to 550 g by
DHEA without affecting food intake. Schwartz (Cancer 39: 1129-1132,
20 1979) observed that C3H mice given DHEA (450 mg/kg, 3 times a week)
gained significantly less weight and grew older than the control animals,
had less body fat and were more active. The reduction in body weight
was achieved without loss of appetite or food restriction. Furthermore,
DHEA could prevent weight gain in animals bred to become obese in
25 adulthood (in Kent, Geriatrics 37: 157-160, 1982)!

DHEA administration to lean Zucker rats decreased body weight gain
despite increased food intake. Treated animals had smaller fat pads thus,
overall, suggesting that DHEA increases food metabolism, resulting in
30 lower weight gain and fat accumulation (Svec et al., Proc. 2nd Int. Conf.,
Cortisol and Anti-Cortisols, Las Vegas, Nevada, USA, p. 56 abst., 1997).

5 Obesity was found to be improved in the A^y mutant mouse (Yen et al.,
Lipids 12: 409-413, 1977) and in the Zucker rat (Cleary and Zisk, Fed.
Proc. 42: 536, 1983). DHEA-treated C3H mice had a younger appearance
than controls (Schwartz, Cancer Res. 39: 1129-1132, 1979).

10 DHEA reduced the incidence of atherosclerosis in cholesterol-fed rabbits
(Gordon et al., J. Clin. Invest. 82: 712-720, 1988; Arad et al.,
Arteriosclerosis 9: 159-166, 1989). Moreover, high serum concentrations
of DHEA-S have been reported to protect against death from
cardiovascular diseases in men (Barrett-Connor et al., N. Engl. J. Med.
15 315: 1519-1524, 1986). Circulating levels of DHEA and DHEA-S have
thus been found to be inversely correlated with mortality from
cardiovascular disease (Barrett-Connor et al., N. Engl. J. Med. 315: 1519-
1524, 1986) and to decrease in parallel with the diminished immune
competence (Thoman and Weigle, Adv. Immunol. 46: 221-222, 1989). A
20 study in man has shown an inverse correlation between fetal serum
DHEA-S and low density lipoprotein (LDL) levels (Parker et al., Science
208: 512, 1980).

25 Uses of DHEA as well as the benefits of androgen and estrogen therapy
are discussed in International Patent Publication WO 94/16709.

Correlations observed in the prior art are not believed to suggest
treatment or prophylactic methods that are effective, or as free of
undesirable side-effects, as are combination therapies disclosed here.

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SUMMARY OF THE INVENTION

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It is accordingly an object of the present invention to provide effective methods of treatment for osteoporosis, hypercholesterolemia, hyperlipidemia, atherosclerosis, breast cancer, endometrial cancer, ovarian cancer and uterine cancer while minimizing undesirable side effects.

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It is another object to provide methods of reducing the risk of acquiring the above diseases.

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It is another object to provide kits and pharmaceutical compositions suitable for use in the above methods.

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In one embodiment, the invention pertains to a method of treating or reducing the risk of acquiring osteoporosis comprising increasing levels of a sex steroid precursor selected from the group consisting of dehydroepiandrosterone (DHEA), dehydroepiandrosterone-sulfate (DHEA-S) and androst-5-ene-3 β ,17 β -diol (5-diol), in a patient in need of said treatment or said reduction, and further comprising administering to said patient a therapeutically effective amount of a selective estrogen receptor modulator (SERM) as part of a combination therapy.

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In another embodiment, the invention provides a method of treating or reducing the risk of acquiring hypercholesterolemia comprising increasing levels of a sex steroid precursor selected from the group consisting of dehydroepiandrosterone, dehydroepiandrosterone-sulfate and androst-5-ene-3 β ,17 β -diol, in a patient in need of said treatment or

5 said reduction, and further comprising administering to said patient a
therapeutically effective amount of a selective estrogen receptor
modulator as part of a combination therapy.

10 In another embodiment, the invention provides a method of treating or
reducing the risk of acquiring hyperlipidemia comprising increasing
levels of a sex steroid precursor selected from the group consisting of
dehydroepiandrosterone, dehydroepiandrosterone-sulfate and androst-
5-ene-3 β ,17 β -diol , in a patient in need of said treatment or said
reduction, and further comprising administering to said patient a
15 therapeutically effective amount of a selective estrogen receptor
modulator as part of a combination therapy.

20 In another embodiment, the invention provides a method of treating or
reducing the risk of acquiring atherosclerosis comprising increasing
levels of a sex steroid precursor selected from the group consisting of
dehydroepiandrosterone, dehydroepiandrosterone-sulfate and androst-
5-ene-3 β ,17 β -diol , in a patient in need of said treatment or said
reduction, and further comprising administering to said patient a
25 therapeutically effective amount of a selective estrogen receptor
modulator as part of a combination therapy.

30 In another embodiment, the invention provides a method of treating or
reducing the risk of acquiring breast cancer comprising increasing levels
of a sex steroid precursor selected from the group consisting of
dehydroepiandrosterone, dehydroepiandrosterone-sulfate and androst-
5-ene-3 β ,17 β -diol , in a patient in need of said treatment or said
reduction, and further comprising administering to said patient a

5 therapeutically effective amount of a selective estrogen receptor modulator as part of a combination therapy.

10 In another embodiment, the invention provides a method of treating or reducing the risk of acquiring endometrial cancer comprising increasing levels of a sex steroid precursor selected from the group consisting of dehydroepiandrosterone, dehydroepiandrosterone-sulfate and androst-
15 5-ene-3 β ,17 β -diol , in a patient in need of said treatment or said reduction, and further comprising administering to said patient a therapeutically effective amount of a selective estrogen receptor modulator as part of a combination therapy.

20 In another embodiment, the invention provides a method of treating or reducing the risk of acquiring uterine cancer comprising increasing levels of a sex steroid precursor selected from the group consisting of dehydroepiandrosterone, dehydroepiandrosterone-sulfate and androst-
25 5-ene-3 β ,17 β -diol , in a patient in need of said treatment or said reduction, and further comprising administering to said patient a therapeutically effective amount of a selective estrogen receptor modulator as part of a combination therapy.

30 In another embodiment, the invention provides a method of treating or reducing the risk of acquiring ovarian cancer comprising increasing levels of a sex steroid precursor selected from the group consisting of dehydroepiandrosterone, dehydroepiandrosterone-sulfate and androst-
35 5-ene-3 β ,17 β -diol , in a patient in need of said treatment or said reduction, and further comprising administering to said patient a

5 therapeutically effective amount of a selective estrogen receptor modulator as part of a combination therapy.

10 In another embodiment, the invention provides a kit comprising a first container containing a therapeutically effective amount of at least one sex steroid precursor selected from the group consisting of dehydroepiandrosterone, dehydroepiandrosterone-sulfate, androst-5-ene-3 β ,17 β -diol and any prodrug that is converted *in vivo* into any of the foregoing precursors; and further comprising a second container containing a therapeutically effective amount of at least one selective estrogen receptor modulator.

15 In another embodiment, the invention provides a pharmaceutical composition comprising: a) a pharmaceutically acceptable excipient, diluent or carrier; b) a therapeutically effective amount of at least one sex steroid precursor selected from the group consisting of dehydroepiandrosterone, dehydroepiandrosterone-sulfate, androst-5-ene-3 β ,17 β -diol and a prodrug that is converted *in vivo* into any of the foregoing sex steroid precursors; and c) a therapeutically effective amount of at least one selective estrogen receptor modulator.

25 As used herein, a selective estrogen receptor modulator (SERM) is a compound that either directly or through its active metabolite functions as an estrogen receptor antagonist ("antiestrogen") in breast tissue, yet provides estrogenic or estrogen-like effect on bone tissue and on serum cholesterol levels (i.e. by reducing serum cholesterol). Non-steroidal compounds that function as estrogen receptor antagonists *in vitro* or in human or rat breast tissue (especially if the compound acts as an

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5 antiestrogen on human breast cancer cells) is likely to function as a
SERM. Conversely, steroidal antiestrogens tend not to function as
SERMs because they tend not to display any beneficial effect on serum
cholesterol. Non-steroidal antiestrogens we have tested and found to
10 function as SERMs include EM-800, EM-01538, Raloxifene, Tamoxifen
and Droloxifene. We have tested the steroidal antiestrogen ICI 182,780
and found not to function as SERMs. SERMs in accordance with the
invention may be administered in the same dosage as known in the art
when these compounds are used as antiestrogens.

15 We have also noted a correlation between the beneficial effect SERMs
have on serum cholesterol and beneficial estrogenic or estrogen-like
effects on bone and on serum lipids. SERMs that have been shown in our
research to act beneficially on all of these parameters, include bone mass,
cholesterol, and triglyceride levels. Without intending to be bound by
20 theory, it is believed that SERMs, many of which preferably, have two
aromatic rings linked by one to two carbon atoms, are expected to
interact with the estrogen receptor by virtue of the foregoing portion of
the molecule that is best recognized by the receptor. Preferred SERMs
have side chains which may selectively cause antagonistic properties in
25 breast tissue without having significant antagonistic properties in other
tissues. Thus, the SERMs may desirably functions as antiestrogens in the
breast while surprisingly and desirably functioning as estrogens (or
providing estrogen-like activity) in bone and in the blood (where
concentrations of lipid and cholesterol are favorably affected). The
30 favorable effect on cholesterol and lipid translates to a favorable effect
against atherosclerosis which is known to be adversely, affected by
improper levels of cholesterol and lipid.

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All of the diseases treated by the invention as discussed herein respond favorably to androgens. Rather than utilizing androgens per se, applicants utilize sex steroid precursors such as DHEA, DHEA-S, 5-diol, or prodrugs converted to any such sex steroid precursors. *In vivo*, DHEA-S is converted to DHEA which in turn converts to 5-diol. It is believed that any tissue responding favorably to one is likely to respond favorably to the others. Prodrug forms of active metabolites are well known in the art. See, e.g. H. Bundgaard "Design and Application of Prodrugs" (In: A Textbook of Drug Design and Development. Edited by H. Bundgaard and P. Krosgaard-Larsen; Harwood Academic Publishers GmbH, Chur, Switzerland, 1991), the contents of which are incorporated herein by reference. In particular, see pages 154-155 describing various functional groups of active metabolites and appropriate corresponding prodrug groups that convert *in vivo* to each functional group. Where a patients' levels of sex steroid precursors are raised in accordance with the invention, that may typically be accomplished by administering such a precursor or by administering a prodrug of such a precursor. By utilizing precursors instead of androgens, undesirable androgenic activity in tissues other than the target is reduced. Tissues convert precursors such as DHEA to androgens only through a natural and more regulated process. A large percentage of androgens are locally produced in peripheral tissues and to different extents in different tissues.

The cancers treated in accordance with the invention respond adversely to estrogenic activity. On the other hand, osteoporosis, hypercholesterolemia, hyperlipidemia, and atherosclerosis respond favorably to estrogenic or estrogen-like activity. By using SERMs in

5 accordance with the invention, desirable effects are provided in target
tissues without undesirable effects in certain other tissues. For example,
a SERM can have favorable estrogenic effect in the bone (or on lipid or
cholesterol) while avoiding unfavorable estrogenic effect in the breast.

10 Thus both precursor and SERM provide favorable effect in target tissues
while minimizing unfavorable effects in certain other tissues. Moreover,
there are substantial synergies in using the two together in accordance
with the invention. For example, estrogens and androgens provide
15 beneficial effect against osteoporosis by different mechanisms (estrogen
reducing bone resorption, androgen contributing to bone formation).
The combination of the present invention provides bone with beneficial
estrogen or estrogen-like effect through the activity of SERM, and also
provides beneficial androgen through local conversion of precursor to
androgen in the bone. Precursor is also believed to provide estrogen.
20 The same is true in connection with controlling lipid or cholesterol
(useful for treating or preventing atherosclerosis). A similar synergy is
provided against breast, endometrial, ovarian or uterine cancer where
the SERM provides desirable antiestrogenic effect and the precursor
provides desirable androgenic effect (with any incidental conversion of
25 precursor to estrogen being mitigated by the antiestrogen). Undesirable
effects are also mitigated in a synergistic way by the combination used in
the invention. For all diseases discussed herein, any other effect on
breast tissues that might otherwise result from estrogens produced by the
precursor (when the precursor is used for promoting androgenic effects
30 in accordance with the invention) is mitigated by the antiestrogenic effect
of the SERM in breast tissue.

5 In some embodiments, progestins are added to provide further androgenic effect. Progestins may be used at low dosages known in the art without adversely affecting receptors other than the androgen receptors (e.g. glucocorticoid receptors). They also are relatively free of
10 unwanted androgenic side effects (such as facial hair with female patients).

Preferred SERMs discussed herein relate: (1) to all diseases stated to be susceptible to the invention; (2) to both therapeutic and prophylactic applications; and (3) to preferred pharmaceutical compositions and kits.

15 In one embodiment, the precursor is DHEA.

In another embodiment, the precursor is DHEA-S.

20 In another embodiment, the precursor is 5-diol.

A patient in need of treatment or of reducing the risk of onset of a given disease is one who has either been diagnosed with such disease or one who is susceptible to acquiring such disease.

25 Except where otherwise stated, the preferred dosage of the active compounds (concentrations and modes of administration) of the invention is identical for both therapeutic and prophylactic purposes. The dosage for each active component discussed herein is the same
30 regardless of the disease being treated (or of the disease whose likelihood of onset is being reduced).

5 Except when otherwise noted or where apparent from context, dosages
herein refer to weight of active compounds unaffected by pharmaceutical
excipients, diluents, carriers or other ingredients, although such
additional ingredients are desirably included, as shown in the examples
herein. Any dosage form (capsule, tablet, injection or the like) commonly
10 used in the pharmaceutical industry is appropriate for use herein, and
the terms "excipient", "diluent", or "carrier" include such nonactive
ingredients as are typically included, together with active ingredients in
such dosage forms in the industry. For example, typical capsules, pills,
enteric coatings, solid or liquid diluents or excipients, flavorants,
15 preservatives, or the like may be included.

All of the active ingredients used in any of the therapies discussed herein
may be formulated in pharmaceutical compositions which also include
one or more of the other active ingredients. Alternatively, they may each
20 be administered separately but sufficiently simultaneous in time so that a
patient eventually has elevated blood levels or otherwise enjoys the
benefits of each of the active ingredients (or strategies) simultaneously.
In some preferred embodiments of the invention, for example, one or
more active ingredients are to be formulated in a single pharmaceutical
composition. In other embodiments of the invention, a kit is provided
25 which includes at least two separate containers wherein the contents of at
least one container differs, in whole or in part, from the contents of at
least one other container with respect to active ingredients contained
therein.

30 Combination therapies discussed herein also include use of one active
ingredient (of the combination) in the manufacture of a medicament for

5 the treatment (or risk reduction) of the disease in question where the
treatment or prevention further includes another active ingredient of the
combination in accordance with the invention. For example in one
embodiment, the invention provides the use of a SERM in the
preparation of a medicament for use, in combination with a sex steroid
10 precursor selected from the group consisting of DHEA, DHEA-S, 5-diol,
and pro-drugs converted to any of the foregoing sex steroid precursors,
in vivo, in the treatment of any of the diseases for which the present
combination therapy is believed effective (i.e., breast cancer, endometrial
cancer, uterine cancer, ovarian cancer, osteoporosis,
15 hypercholesterolemia, hyperlipidemia, and atherosclerosis). In another
embodiment, the invention provides the use of a sex steroid precursor
selected from the group consisting of DHEA, DHEA-S, 5-diol, and pro-
drugs converted to any of the foregoing sex steroid precursors, *in vivo*, in
the preparation of a medicament for use, in combination with a SERM,
20 for treatment of any of those same diseases.

In one embodiment of the invention, DHEA is not utilized as the
precursor. In another embodiment, EM-800 is not used as the SERM. In
another embodiment, the combination of DHEA with EM-800 is not used.

25 In one preferred embodiment, DHEA is used in combination with
EM-1538.

BRIEF DESCRIPTION OF THE DRAWINGS

Figure 1 shows the effect of treatment with DHEA (10 mg, percutaneously, once daily) or EM-800 (75 µg, orally, once daily) alone or in combination for 9 months on the incidence of DMBA-induced mammary carcinoma in the rat throughout the 279-day observation period. Data are expressed as percentage of the total number of animals each group.

Figure 2 shows the effect of treatment with DHEA (10 mg, percutaneously, once daily) or EM-800 (75 µg, orally, once daily) alone or in combination for 9 months on average tumor number per tumor-bearing animal (A) and on average tumor size per tumor-bearing rat (B) throughout the 279-day observation period. Data are expressed as the means ± SEM.

Figure 3 shows the effect of treatment with DHEA (10 mg, percutaneously, once daily) or EM-800 (75 µg, orally, once daily) alone or in combination for 9 months on serum triglyceride (A) and cholesterol (B) levels in the rat. Data are expressed as the means ± SEM. **: $P < 0.01$ experimental versus respective control.

Figure 4 shows: A) Effect of increasing doses of DHEA (0.3 mg, 1.0 mg or 3.0 mg) administered percutaneously twice daily on average ZR-75-1 tumor size in ovariectomized (OVX) nude mice supplemented with estrone. Control OVX mice receiving the vehicle alone are used as additional controls. The initial tumor size was taken as 100%. DHEA was administered percutaneously (p.c.) in a 0.02 ml solution of 50% ethanol -

5 50% propylene glycol on the dorsal skin. B) Effect of treatment with increasing doses of DHEA or EM-800 alone or in combination for 9.5 months on ZR-75-1 tumor weight in OVX nude mice supplemented with estrone. **, $p < 0.01$, treated versus control OVX mice supplemented with estrone.

10 Figure 5 shows the effect of increasing oral doses of the antiestrogen EM-800 (15 μ g, 50 μ g or 100 μ g) (A) or of percutaneous administration of increasing doses of DHEA (0.3, 1.0 or 3.0 mg) combined with EM-800 (15 μ g) or EM-800 alone (B) for 9.5 months on average ZR-75-1 tumor size in
15 ovariectomized(OVX) nude mice supplemented with estrone. The initial tumor size was taken as 100%. Control OVX mice receiving the vehicle alone were used as additional controls. Estrone was administered subcutaneously at the dose of 0.5 μ g once daily while DHEA was dissolved in 50% ethanol - 50% propylene glycol and applied on the
20 dorsal skin area twice daily in a volume of 0.02 ml. Comparison is also made with OVX animals receiving the vehicle alone.

25 Figure 6 shows the effect of 12-month treatment with dehydroepiandrosterone (DHEA) alone or in combination with Flutamide or EM-800 on trabecular bone volume in ovariectomized rats. Intact animals are added as additional controls. Data are presented as mean \pm SEM ** $p < 0.01$ versus OVX Control.

30 Figure 7 shows the effect of 12-month treatment with dehydroepiandrosterone (DHEA) alone or in combination with Flutamide or EM-800 on trabecular number in ovariectomized rats. Intact

5 animals are added as additional controls. Data are presented as mean \pm SEM ** $p < 0.01$ versus OVX Control.

10 Figure 8 shows proximal tibia metaphyses from intact control (A), ovariectomized control (B), and ovariectomized rats treated with DHEA alone (C) or in combination with Flutamide (D) or EM-800 (E). Note the reduced amount of trabecular bone (T) in ovariectomized control animals (B), and the significant increase in trabecular bone volume (T) induced after DHEA administration (C). The addition of Flutamide to DHEA partially blocked the effect of DHEA on the trabecular bone volume (D),
15 whereas the combination of DHEA and EM-800 provided complete protection against the ovariectomy-associated bone loss. Modified trichrome Masson-Goldner, magn.x80. T: Trabeculae, GP: Growth Plate.

20 Figure 9 shows the effect of increasing doses (0.01, 0.03, 0.1, 0.3, and 1 mg/kg) of EM-800, EM-1538 and Raloxifene (EM-1105) administered per os daily for 4 days on cholesterol level of ovariectomized rat.

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DETAIL DESCRIPTION OF THE INVENTION

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Estrogens are well-known to stimulate the proliferation of breast epithelial cells and cell proliferation itself is thought to increase the risk of cancer by accumulating random genetic errors that may result in neoplasia (Preston Martin et al., Cancer. Res. 50: 7415-21, 1990).⁷ Based on this concept, antiestrogens have been introduced to prevent breast cancer with the objective of reducing the rate of cell division stimulated by estrogens.

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The loss of ovarian cyclicity found in female Sprague-Dawley rats after 10 months of age is accompanied by increased serum estrogen and prolactin levels and decreased serum androgen and progesterone concentrations (Lu et al., 61st Annual Meeting of the Endocrine Society, 106 (abst. #134), 1979; Tang et al., Biol. Reprod. 31: 399-413, 1984; Russo et al., Monographs on Pathology of Laboratory Animals: Integument and Mammary Glands 252-266, 1989; Sortino and Wise, Endocrinology 124: 90-96, 1989; Cardy, Vet. Pathol. 28: 139-145, 1991). These hormonal changes that spontaneously occur in aging female rats are associated with multifocal proliferation and increased secretory activity of the acinar/alveolar tissue as well as mammary gland duct dilatation and formation of cysts (Boorman et al., 433, 1990; Cardy, Vet. Pathol. 28: 139-145, 1991). It should be mentioned that hyperplastic and neoplastic changes of the rat mammary gland are often accompanied by increased levels of estrogens and prolactin (Meites, J. Neural. Transm. 48: 25-42, 1980).⁷ Treatment with EM-800, a SERM of the present invention, induces atrophy of the mammary gland which is characterized by a decrease in the size and number of the lobular structures, and no evidence of

5 secretory activity, indicating the potent antiestrogenic activity of EM-800
in the mammary gland (Luo et al. *Endocrinology* 138: 4435-4444, 1997).

10 Treatment with DHEA, a sex steroid precursor of the present invention,
leads to an elevation in serum DHEA and 5-diol while serum 4-dione,
testosterone, dihydrotestosterone, and estradiol levels are only
moderately increased or more often remain unchanged, thus confirming
the intracellular biotransformation of this precursor steroid in peripheral
tissues (Labrie et al., *Mol. Cell. Endocrinol.* 78: C113-C118, 1991).
However, the stimulatory effect of orally administered DHEA on serum
15 androgens, such as testosterone and dihydrotestosterone, is of greater
amplitude than the effect on serum estrogens, thus suggesting that
DHEA is predominantly transformed into androgens in these animals.
This observation is in agreement with the data obtained in women where
the formation of androgens from DHEA was a more important pathway
20 than the conversion of DHEA into estrogens (Morales et al., *J. Clin.*
Endocrinol. Metab. 78: 1360-1367, 1994; Labrie et al., *Ann. N. Y. Acad. Sci.*
774: 16-28, 1995; Labrie et al., *Steroids* 62: 148-158, 1997).

25 With the knowledge of the above-described potent antiestrogenic activity
resulting in mammary gland atrophy and the predominant androgenic
effect of DHEA on the mammary gland, the histomorphological changes
seen in animals treated with the combination of a SERM and a sex steroid
precursor are best explained by an unopposed androgenic action of
DHEA in the rat mammary gland.

30 Most importantly, it has been observed that androgens exert a direct
antiproliferative activity on the growth of ZR-75-1 human breast cancer

5 cells *in vitro* and that such an inhibitory effect of androgens is additive to
that of an antiestrogen (Poulin and Labrie, Cancer Res. 46: 4933-4937, 1986; Poulin et al., Breast Cancer Res. Treat. 12: 213-225, 1988). Similar
10 inhibitory effects have been observed *in vivo* on ZR-75-1 xenografts in
nude mice (Dauvois et al., Cancer Res. 51: 3131-3135, 1991). Androgens
have also been shown to inhibit the growth of DMBA-induced mammary
carcinoma in the rat, this inhibition being reversed by the simultaneous
administration of the pure antiandrogen Flutamide (Dauvois et al., Breast
15 Cancer Res. Treat. 14: 299-306, 1989). Taken together, the present data
indicate the involvement of the androgen receptor in the inhibitory
action of DHEA on breast cancer.

Since antiestrogens and sex steroid precursors exert inhibitory effects on
breast cancer via different mechanisms, the present invention shows that
the combination of a SERM (EM-800) and a sex steroid precursor (DHEA)
20 exerts more potent inhibitory effects than each compound used alone on
the development of DMBA-induced rat mammary carcinoma as well
illustrated in Figures 1 and 2. In fact, no DMBA-induced tumor was
found at the end of the experiment in animals that had received both
DHEA and EM-800.

25 The present invention describes that the combination of a sex steroid
precursor (DHEA) and a SERM (EM-800) maintained the stimulatory
effect of DHEA on bone formation and potentiated the inhibitory effect of
the SERM (EM-800) alone on bone turnover and resorption as
30 demonstrated by the further decreases in urinary hydroxyproline and
calcium excretion when both compounds were combined.

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5 We have shown that DHEA has beneficial effects on bone in both the female rat (Luo et al., *Endocrinology* 138: 4435-4444, 1997), and postmenopausal women (Labrie et al., *J. Clin. Endocrinol. Metab.* 82: 3498-3505, 1997). Thus, in intact female rats, treatment with DHEA increases bone mineral density (BMD) of total skeleton, lumbar spine and femur (Luo et al., *Endocrinology* 138: 4435-4444, 1997).

10 On the other hand, treatment with EM-800 had no significant effect on BMD in intact animals although potent stimulatory effects are observed in the ovariectomized rat (Martel et al., unpublished data). Since EM-800 exerts such stimulatory effects on BMD of total skeleton, lumbar spine and femur in ovariectomized rats, the lack of significant stimulatory effect of EM-800 in intact animals could be due to the fact that the sex steroids present in intact female rats exert maximal effect on BMD (Luo et al., *Endocrinology* 138: 4435-4444, 1997). Similarly, the lack of significant effect of EM-800 in ovariectomized rats already receiving DHEA is likely due to the maximal stimulatory effects exerted by the androgens (and possibly estrogens) synthesized in bone cells from exogenous DHEA.

25 Estrogens are known to lower serum cholesterol but to increase or to have no effect on serum triglycerides levels (Love et al., *Ann. Intern. Med.* 115: 860-864, 1991; Walsh et al., *New Engl. J. Med.* 325: 1196-1204, 1991; Barrett-Connor, *Am. J. Med.* 95 (Suppl. 5A): 40S-43S, 1993; Russell et al., *Atherosclerosis* 100: 113-122, 1993; Black et al., *J. Clin. Invest.* 93: 63-69, 1994; Dipippo et al., *Endocrinology* 136: 1020-1033, 1995; Ke et al., *Endocrinology* 136: 2435-2441, 1995). Figure 3 shows that EM-800 possesses both hypocholesterolemic and hypotriglyceridemic effects in

- 25 -

5 the rat, thus showing its unique action on the serum lipid profile which is
apparently different from other SERMs, such as tamoxifen (Bruning et al.,
Br. J. Cancer 58: 497-499, 1988; Love et al., J. Natl. Cancer Inst. 82: 1327-
1332, 1990; Dipippo et al., Endocrinology 136: 1020-1033, 1995; Ke et al.,
Endocrinology 136: 2435-2441, 1995), droloxifene (Ke et al.,
10 Endocrinology 136: 2435-2441, 1995), and raloxifene (Black et al., J. Clin.
Invest. 93: 63-69, 1994). The combination of DHEA and EM-800
preserved the hypocholesterolemic and hypotriglyceridemic effects of
EM-800, thus suggesting that such a combination could exert beneficial
effects on serum lipids.

15 It should be mentioned that the serum lipid profile is markedly different
between rats and humans. However, since an estrogen receptor-
mediated mechanism is involved in the hypocholesterolemic effect of
estrogens as well as antiestrogens (Lundeen et al., Endocrinology 138:
20 1552-1558, 1997), the rat remains a useful model to study the cholesterol-
lowering effect of estrogens and "antiestrogens" in humans.

In brief, the above-described data clearly demonstrate the effects of the
combination of a SERM (EM-800) and a sex steroid precursor (DHEA) on
25 the development of mammary carcinoma induced by DMBA as well as
the protective effects of such a combination on bone mass and serum
lipids. Such data suggest the additional beneficial effects of such a
combination for treatment and prevention of osteoporosis while
improving the lipid profile.

30

We have also studied the potential interaction of the inhibitory effect of
the novel antiestrogen (EM-800) with that of sex steroid precursor

5 (DHEA) on the growth of human ZR-75-1 breast cancer xenografts in
nude mice by combined administration of the two drugs. Figures 4 and 5
show that DHEA, by itself, at the doses used, causes a 50 to 80%
inhibition of tumor growth while the near complete inhibition of tumor
growth achieved with a low dose of the antiestrogen was not affected by
10 DHEA.

The limitations of bone mineral density (BMD) measurements are well
known. As an example, BMD measurements showed no change in rats
treated with the steroidal antiestrogen ICI 182780 (Wakeling, *Breast*
15 *Cancer Res. Treat.* 25: 1-9, 1993) while inhibitory changes were seen by
histomorphometry (Gallagher et al., *Endocrinology* 133: 2787-2791, 1993).
Similar differences were reported with Tamoxifen (Jordan et al., *Breast* 9
Cancer Res. Treat. 10: 31-35, 1987; Sibonga et al., *Breast Cancer Res.*
Treatm. 41: 71-79, 1996).

20 It should be indicated that reduced bone mineral density is not the only
abnormality associated with reduced bone strength. (Guidelines for
preclinical and clinical evaluation of agents used in the prevention or
treatment of postmenopausal osteoporosis, Division of Metabolism and
25 Endocrine Drug Products, FDA, May 1994). It is thus important to
analyze the changes in biochemical parameters of bone metabolism
induced by various compounds and treatments in order to gain a better
knowledge of their action.

30 It is particularly important to indicate that the combination of DHEA and
EM-800 exerted unexpected beneficial effects on important biochemical
parameters of bone metabolism. In fact, DHEA alone did not affect the

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5 urinary hydroxyproline/creatinine ratio, a marker of bone resorption. Moreover, no effect of DHEA could be detected on daily urinary calcium or phosphorus excretion (Luo et al., Endocrinology 138: 4435-4444, 1997).
10 EM-800, on the other hand, decreased the urinary hydroxyproline/creatinine ratio by 48% while, similarly to DHEA, no effect of EM-800 was seen on urinary calcium or phosphorus excretion. EM-800, moreover, had no effect on serum alkaline phosphatase activity, a marker of bone formation while DHEA increased the value of the parameter by about 75% (Luo et al., Endocrinology 138: 4435-4444, 1997).

15 One of the unexpected effects of the combination of DHEA and EM-800 relates to the urinary hydroxyproline/creatinine ratio, a marker of bone resorption, which was reduced by 69% when both DHEA and EM-800 were combined, this value being statistically different ($p < 0.01$) from the 48% inhibition achieved by EM-800 alone while DHEA alone did not
20 show any effect. Thus, the addition of DHEA to EM-800 increases by 50% of the inhibitory effect of EM-800 on bone reabsorption. Most importantly, another unexpected effect of the addition of DHEA to EM-800 was the approximately 84% decrease in urinary calcium (from 23.17 ± 1.55 to 3.71 ± 0.75 $\mu\text{mol}/24\text{h}/100\text{g}$ ($p < 0.01$) and the 55% decrease in
25 urinary phosphorus (from 132.72 ± 6.08 to 59.06 ± 4.76 $\mu\text{mol}/24\text{h}/100\text{g}$ ($p < 0.01$) respectively, (Luo et al., Endocrinology 138: 4435-4444, 1997). ¶

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Table 1

| GROUP | URINE | | | SERUM |
|----------------------------|---|--|--|-------------------------|
| | CALCIUM ($\mu\text{mol}/24\text{h}/100\text{g}$) | PHOSPHORUS ($\mu\text{mol}/24\text{h}/100\text{g}$) | HP/Cr ($\mu\text{mol}/\text{mmol}$) | tALP (IU/L) |
| CONTROL | 23.17 ± 1.55 | 132.72 ± 6.08 | 13.04 ± 2.19 | 114.25 ± 14.04 |
| DHEA (10 mg) | 25.87 ± 3.54 | 151.41 ± 14.57 | 14.02 ± 1.59 | $198.38 \pm 30.76^*$ |
| EM-800 (75 μg) | 17.44 ± 4.5 | 102.03 ± 25.13 | $6.81 \pm 0.84^{**}$ | 114.11 ± 11.26 |
| DHEA + EM-800 | $3.71 \pm 0.75^{**}$ | $59.06 \pm 4.76^{**}$ | $4.06 \pm 0.28^{**}$ | $204.38 \pm 14.20^{**}$ |

10 It is also of interest to note that the potent inhibitory effect of EM-800 on serum cholesterol is not prevented by simultaneous treatment with DHEA (Luo et al., Endocrinology 138: 4435-4444, 1997).

15 While Raloxifene and similar compounds prevent bone loss and decrease serum cholesterol (like estrogens), it should be mentioned that when Raloxifene was compared to Premarin on BMD, the effect of Raloxifene on BMD was less potent than that of Premarin (Minutes of the
 20 Endocrinology and Metabolism Drugs Advisory Committee, FDA Thursday, Meeting #68, November 20th 1997). Because of its well known adverse effects on breast and uterine cancer, the addition of an estrogen to Raloxifene, EM-800 or other similar compounds is not an acceptable solution.

25 The present results obtained in the rat clearly demonstrate that DHEA can provide the beneficial effects which are lacking with the use of a selective estrogen receptor modulator (SERM) alone such as EM-800, Raloxifene, etc. While a SERM has effects limited to inhibition of bone

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5 resorption, the addition of DHEA, 5-diol, DHEA-S is believed to stimulate bone formation (an effect not found with a SERM or an estrogen) and further reduce bone resorption above the effect achieved with EM-800.

10 Importantly, the combination of EM-800 and DHEA in ovariectomized rats treated for 12 months has beneficial effects on bone morphometry. Trabecular bone volume is particularly important for bone strength and to prevent bone fractures. Thus, in the above-mentioned study, trabecular bone volume of the tibia increased from $4.1 \pm 0.7\%$ in
15 ovariectomized rats to $11.9 \pm 0.6\%$ ($p < 0.01$) with DHEA alone while the addition of EM-800 to DHEA further increased trabecular bone volume to $14.7 \pm 1.4\%$, a value similar to that found in intact controls (Fig. 6)

From a value of 0.57 ± 0.08 per mm in ovariectomized rats, treatment
20 with DHEA resulted in a 137% increase in trabecular bone number compared to ovariectomized controls. The stimulatory effect of DHEA thus reached 1.27 ± 0.1 per mm while simultaneous treatment with EM-800 and DHEA resulted in an additional 28% increase in trabecular bone number ($p < 0.01$) compared to that achieved by DHEA alone
25 (Fig. 7). Similarly, the addition of EM-800 to DHEA treatment, resulted in an additional 15% ($p < 0.05$) decrease in trabecular bone separation, compared to that achieved with DHEA alone, thus leading to values not different from those seen in intact controls.

30 As complement to the numerical data presented in figures 6 and 7, figure 8 illustrates the increase in trabecular bone volume in the proximal tibia metaphysis induced by DHEA in ovariectomized treated animals (C)

- 30 -

5 compared to ovariectomized controls (B), as well as the partial inhibition
of the stimulatory effect of DHEA after the addition of Flutamide to
DHEA treatment (D). On the other hand, administration of DHEA in
combination with EM-800 resulted in a complete prevention of the
10 ovariectomy-induced osteopenia (E), the trabecular bone volume being
comparable to that seen in intact controls (A).

The bone loss observed at menopause in women is believed to be related
to an increase in the rate of bone resorption which is not fully
compensated by the secondary increase in bone formation. In fact, the
15 parameters of both bone formation and bone resorption are increased in
osteoporosis and both bone resorption and formation are inhibited by
estrogen replacement therapy. The inhibitory effect of estrogen
replacement on bone formation is thus believed to result from a coupled
mechanism between bone resorption and bone formation, such that the
20 primary estrogen-induced reduction in bone resorption entrains a
reduction in bone formation (Parfitt, *Calcified Tissue International* 36
Suppl. 1: S37-S45, 1984).

Cancellous bone strength and subsequent resistance to fracture do not
25 only depend upon the total amount of cancellous bone but also on the
trabecular microstructure, as determined by the number, size, and
distribution of the trabeculae. The loss of ovarian function in
postmenopausal women is accompanied by a significant decrease in total
trabecular bone volume (Melsen et al., *Acta Pathologica & Microbiologica*
30 *Scandinavia* 86: 70-81, 1978; Vakamatsou et al., *Calcified Tissue*
International 37: 594-597, 1985), mainly related to a decrease in the

5 number and, to a lesser degree, in the width of trabeculae (Weinstein and
Hutson, Bone 8: 137-142, 1987).

In the present study, the androgenic stimulatory effect of DHEA was
observed on almost all the bone histomorphometric parameters studied.
10 DHEA thus resulted in a significant increase in trabecular bone volume
as well as trabecular number, while it decreased the intertrabecular area.

In order to facilitate the combination therapy aspect of the invention, for
any indication discussed herein, the invention contemplates
15 pharmaceutical compositions which include both the SERM or the
bisphosphonate compound and the sex steroid precursor (DHEA ,
DHEAS, 5-diol) in a single composition for simultaneous administration.
The composition may be suitable for administration in any traditional
manner including but not limited to oral administration, subcutaneous
20 injection, intramuscular injection or percutaneous administration. In
other embodiments, a kit is provided wherein the kit includes one or
more SERM or bisphosphonate and sex steroid precursors in separate or
in one container. The kit may include appropriate materials for oral
administration, e.g. tablets, capsules, syrups and the like and for
25 transdermal administration, e.g., ointments, lotions, gels, creams,
sustained release patches and the like.

Applicants believe that administration of SERMs and sex steroid
precursors has utility in the treatment and/or prevention of the
30 development of osteoporosis, breast cancer, hypercholesterolemia,
hyperlipidemia or atherosclerosis. The active ingredients of the

5 invention (whether SERM or precursor or bisphosphonate or otherwise)
may be formulated and administered in a variety of manner.

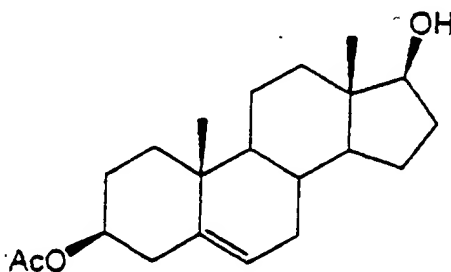
10 Active ingredient for transdermal or transmucosal is preferably present
at from 0.5% to 20% by weight relative to the total weight of the
pharmaceutical composition more preferably between 2 and 10%. DHEA
or 5-diol should be at a concentration of at least 7% for percutaneous
administration. Alternatively, the active ingredient may be placed into a
transdermal patch having structures known in the art, for example,
structures such as those set forth in E.P. Patent No. 0279982.

15 When formulated as an ointment, lotion, gel or cream or the like, the
active compound is admixed with a suitable carrier which is compatible
with human skin or mucosa and which enhances transdermal
penetration of the compound through the skin or mucosa. Suitable
20 carriers are known in the art and include but are not limited to Klucel HF
and Glaxal base. Some are commercially available, e.g., Glaxal base
available from Glaxal Canada Limited Company. Other suitable vehicles
can be found in Koller and Buri, S.T.P. Pharma 3(2), 115-124, 1987. The
carrier is preferably one in which the active ingredient(s) is (are) soluble
25 at ambient temperature at the concentration of active ingredient that is
used. The carrier should have sufficient viscosity to maintain the
inhibitor on a localized area of skin or mucosa to which the composition
has been applied, without running or evaporating for a time period
sufficient to permit substantial penetration of the precursor through the
30 localized area of skin or mucosa and into the bloodstream where it will
cause a desirable clinical effect. The carrier is typically a mixture of
several components, e.g. pharmaceutically acceptable solvents and a

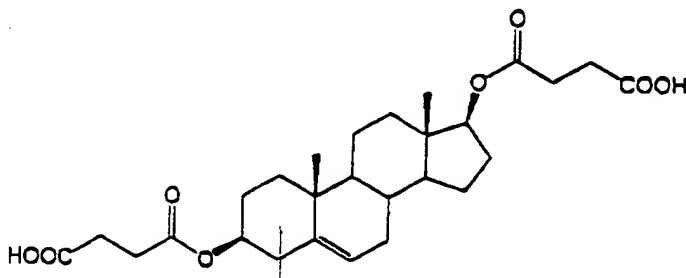
thickening agent. A mixture of organic and inorganic solvents can aid hydrophylic and lipophylic solubility, e.g. water and an alcohol such as ethanol.

Preferred sex steroid precursors are dehydroepiandrosterone (DHEA) (available from Diosynth Inc., Chicago, Illinois, USA), its prodrugs (available from Steraloids, Wilton, New Hampshire, USA), 5-androsten-3 β ,17 β -diol and its prodrugs EM-1304 and EM-01474-D (available from Steraloids, Wilton, New Hampshire USA).

EM-1304



EM-01474-D



It is preferred that the sex steroid precursor is formulated as an alcoholic gel containing 2.0 to 10% of caprylic-capric triglyceride (Neobee M-5); 10 to 20% of hexylene glycol; 2.0 to 10% of diethyleneglycol monomethyl ether (Transutol); 2.0 to 10% of Cyclomethicone (Dow Corning 345); 1.0 to 2% of benzyl alcohol and 1.0 to 5.0% of hydroxypropylcellulose (Klucel HF).

5 The carrier may also include various additives commonly used in
ointments and lotions and well known in the cosmetic and medical arts.
For example, fragrances, antioxidants, perfumes, gelling agents,
thickening agents such as carboxymethylcellulose, surfactants, stabilizers,
emollients, coloring agents and other similar agents may be present.
10 When used to treat systemic diseases, the site of application on the skin
should be changed in order to avoid excess local concentration of active
ingredient and possible overstimulation of the skin and sebaceous glands
by androgenic metabolites of sex steroid precursor.

15 In a pharmaceutical composition for oral administration, DHEA or other
precursor is preferably present in a concentration between 5 and 98% by
weight relative to total weight of the composition more preferably
between 50 and 98 percent, especially between 80 and 98 percent. A
single precursor such as DHEA may be the only active ingredient, or
20 alternatively, a plurality of precursors and/or their analogues may be
used (e.g., a combination of DHEA, DHEA-S, 5-diol, or a combination of
two or more compounds converted *in vivo* to DHEA, DHEA-S or 5-diol
or a combination of DHEA or 5-diol and one or more analogues thereof
which are converted to DHEA or 5-diol *in vivo*, etc. The blood level of
25 DHEA is the final criteria of adequate dosage which takes into account
individual variation in absorption and metabolism.

Preferably, the attending clinician will, especially at the beginning of
treatment, monitor an individual patient's overall response and serum
30 levels of DHEA (in comparison to the preferred serum concentrations
discussed above), and monitor the patient's overall response to

5 treatment, adjusting dosages as necessary where a given patients' metabolism or reaction to treatment is atypical.

Treatment in accordance with the invention is suitable for indefinite continuation. It is expected that DHEA and/or 5-diol treatment will
10 simply maintain DHEA levels within a range similar to that which occurs naturally in women before menopause (serum concentration between 4 and 10 micrograms per liter), or naturally in young adult men (serum concentration between 4 and 10 micrograms per liter).

15 The SERM compound or bisphosphonate and/or the sex steroid precursor can also be administered, by the oral route, and may be formulated with conventional pharmaceutical excipients, e.g. spray dried lactose, microcrystalline cellulose, and magnesium stearate into tablets or capsules for oral administration.

20 The active substance can be worked into tablets or dragee cores by being mixed with solid, pulverulent carrier substances, such as sodium citrate, calcium carbonate or dicalcium phosphate, and binders such as polyvinyl pyrrolidone, gelatin or cellulose derivatives, possibly by adding also
25 lubricants such as magnesium stearate, sodium lauryl sulfate, "Carbowax" or polyethylene glycol. Of course, taste-improving substances can be added in the case of oral administration forms.

30 As further forms, one can use plug capsules, e.g. of hard gelatin, as well as closed self-gelatin capsules comprising a softner or plasticizer, e.g. glycerine. The plug capsules contain the active substance preferably in the form of granulate, e.g. in mixture with fillers, such as lactose,

5 saccharose, mannitol, starches, such as potato starch or amylopectin, cellulose derivatives or highly dispersed silicic acids. In self-gelatin capsules, the active substance is preferably dissolved or suspended in suitable liquids, such as vegetable oils or liquid polyethylene glycols.

10 The lotion, ointment, gel or cream should be thoroughly rubbed into the skin so that no excess is plainly visible, and the skin should not be washed in that region until most of the transdermal penetration has occurred preferably at least 4 hours and, more preferably, at least 6 hours.

15 A transdermal patch may be used to deliver precursor in accordance with known techniques. It is typically applied for a much longer period, e.g., 1 to 4 days, but typically contacts active ingredient to a smaller surface area, allowing a slow and constant delivery of active ingredient.

20 A number of transdermal drug delivery systems that have been developed, and are in use, are suitable for delivering the active ingredient of the present invention. The rate of release is typically controlled by a matrix diffusion, or by passage of the active ingredient
25 through a controlling membrane.

30 Mechanical aspects of transdermal devices are well known in the art, and are explained, for example, in United States Patents 5,162,037, 5,154,922, 5,135,480, 4,666,441, 4,624,665, 3,742,951, 3,797,444, 4,568,343, 5,064,654, 5,071,644, 5,071,657, the disclosures of which are incorporated herein by reference. Additional background is provided by European Patent 0279982 and British Patent Application 2185187.

5

10

The device may be any of the general types known in the art including adhesive matrix and reservoir-type transdermal delivery devices. The device may include drug-containing matrixes incorporating fibers which absorb the active ingredient and/or carrier. In a reservoir-type device, the reservoir may be defined by a polymer membrane impermeable to the carrier and to the active ingredient.

15

In a transdermal device, the device itself maintains active ingredient in contact with the desired localized skin surface. In such a device, the viscosity of the carrier for active ingredient is of less concern than with a cream or gel. A solvent system for a transdermal device may include, for example, oleic acid, linear alcohol lactate and dipropylene glycol, or other solvent systems known in the art. The active ingredient may be dissolved or suspended in the carrier.

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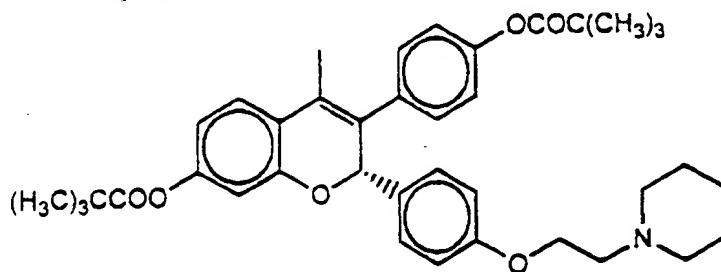
For attachment to the skin, a transdermal patch may be mounted on a surgical adhesive tape having a hole punched in the middle. The adhesive is preferably covered by a release liner to protect it prior to use. Typical material suitable for release includes polyethylene and polyethylene-coated paper, and preferably silicone-coated for ease of removal. For applying the device, the release liner is simply peeled away and the adhesive attached to the patient's skin. In United States Patent 5,135,480, the disclosure of which is incorporated by reference, Bannon et al. describe an alternative device having a non-adhesive means for securing the device to the skin.

30

5 The percutaneous or transmucosal delivery system of the invention may also be used as a novel and improved delivery system for the prevention and/or treatment of osteoporosis or other diseases which respond favorably to treatment with androgens and/or estrogens.

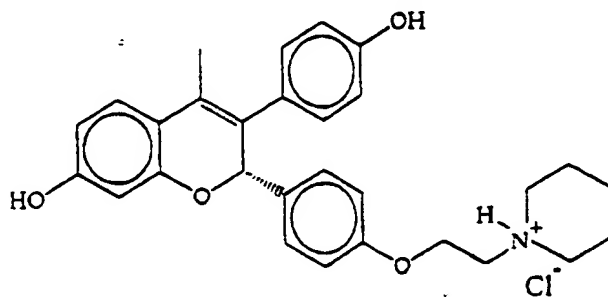
10 A selective estrogen receptor modulator of the invention has a molecular formula with the following features: a) two aromatic rings spaced by 1 to 2 intervening carbon atoms, both aromatic rings being either unsubstituted or substituted by a hydroxyl group or a group converted *in vivo* to hydroxyl; and b) a side chain possessing an aromatic ring and a tertiary amine function or salt thereof.

15 One preferred SERM of the invention is EM-800 reported in PCT/CA96/00097 (WO 96/26201). The molecular structure of EM-800 is:



20

Another preferred SERM of the invention is EM-01538 :



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5 Other preferred SERMs of the invention include Tamoxifen ((Z)-2-[4-(1,2-
diphenyl-1-^{butenyl}phenyl)]-N,N-dimethylethanamine) (available from Zeneca,
UK), Toremifene (available from Orion-Farmos Pharmaceutica, Finland,
or Schering-Plough), Droloxifene and CP-336,156 (cis-1R-[4'-pyrrolidino-
ethoxyphenyl]-2S-phenyl-6-hydroxy-1,2,3,4-tetrahydronaphthalene D-(-)-
10 tartrate salt) (Pfizer Inc., USA), Raloxifene (Eli Lilly and Co., USA), LY
335563 and LY 353381 (Eli Lilly and Co., USA), Iodoxifene (SmithKline
Beecham, USA), Levormeloxifene (3,4-trans-2,2-dimethyl-3-phenyl-4-[4-
(2-(2-(pyrrolidin-1-yl)ethoxy)phenyl]-7-methoxychroman) (Novo
Nordisk, A/S, Denmark) which is disclosed in Shalmi et al. WO
15 97/25034, WO 97/25035, WO 97/25037, WO 97/25038; and Korsgaard et
al. WO 97/25036), GW5638 (described by Willson et al., Endocrinology,
138(9), 3901-3911, 1997) and indole derivatives (disclosed by Miller et al.
EP-0802183A1) and nonsteroidal estrogen derivatives described in WO
97/32837.

20 Any SERM used as required for efficacy, as recommended by the
manufacturer, can be used. Appropriate dosages are known in the art.
Any other non steroidal antiestrogen commercially available can be used
according to the invention. Any compound having activity similar to
25 SERMs (example: Raloxifene can be used).

SERMs administered in accordance with the invention are preferably
administered in a dosage range between 0.01 to 10 mg/kg of body
weight per day (preferably 0.05 to 1.0 mg/kg), with 5 mg per day,
30 especially 10 mg per day, in two equally divided doses being preferred
for a person of average body weight when orally administered, or in a
dosage range between 0.003 to 3.0 mg/kg of body weight per day

- 40 -

5 (preferably 0.015 to 0.3 mg/ml), with 1.5 mg per day, especially 3.0 mg per day, in two equally divided doses being preferred for a person of average body weight when parentally administered (i.e. intramuscular, subcutaneous or percutaneous administration). Preferably the SERMs are administered together with a pharmaceutically acceptable diluent or
10 carrier as described below.

Preferred bisphosphonates of the invention include Alendronate [(4-amino-1-hydroxybutylidene)bis phosphonic acid, disodium salt, hydrate] available from Merck Shape and Dohme under the Tradename of
15 Fosamax, Etidronate [(1-hydroxyethylidene)bis phosphonic acid, 2,2'-iminobis ethanol] available from Procter and Gamble under the Trade names of Didrocal and Didronel , Clodronate [(dichloromethylene)bis phosphonic acid, disodium salt] available from Rhône-Poulenc Rorer under the Trade name of Bonefos or available from Boehringer
20 Mannheim under the Trade name of Ostac and, Pamidronate (3-amino-1-hydroxypropylidene)bis phosphonic acid, disodium salt) available from Geigy under the Tradename of Aredia. Risedronate (1-hydroxy-2-(3-pyridinyl)ethylidene bisphosphonic acid monosodium salt) is under clinical development. Any other bisphosphonates commercially
25 available can be used according to the invention, all at the manufacturers' recommended dosage. Likewise sex steroid precursors may be utilized at dosages recommended in the prior art, preferably at dosages that restore circulating levels to those of healthy males 20-30 years of age or those of premenopausal adult females.

1. The first step in the process is to identify the problem or issue that needs to be addressed. This involves gathering information and understanding the context of the problem.

5

EXAMPLES

Example 1

MATERIALS AND METHODS

10

Animals

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Female Sprague-Dawley rats [CrI:CD(SD)Br] were obtained at 44-46 days of age from Charles River Canada Inc. (St. Constant, Quebec) and housed 2 per cage in a light (12h light/day; lights on at 07:15h)- and temperature ($22 \pm 2^{\circ}\text{C}$)-controlled environment. Animals received Purina rodent chow and tap water *ad libitum*. The animal studies were conducted in a Canadian Council on Animal Care (CCAC)-approved facility in agreement with the CCAC Guide for Care and Use of Experimental Animals.

20

Induction of mammary tumors by DMBA

25

Mammary carcinomas were induced by a single intragastric administration of 20 mg of DMBA (Sigma Chemical Co., St. Louis, MO) in 1 ml of corn oil at 50-52 days of age. Two months later, tumor measurement was performed biweekly. The two largest perpendicular diameters of each tumor were recorded with calipers to estimate tumor size as described (Asselin et al., *Endocrinology* 101: 666-671, 1977). Tumor site, size and number were recorded.

Treatment

30

The animals were randomly divided into groups each containing 20 rats with the exception of 40 animals in the control group. The animals were treated for 282 days with the following: (1) control vehicles, for both

5 DHEA and EM-800; (2) EM-800 ((+)-7-pivaloyloxy-3-(4'-
pivaloyloxyphenyl)-4-methyl-2-(4''-(2'''-piperidinoethoxy)phenyl)-2H-
benzopyran) (75 µg, orally, once daily) in 0.5 ml of a 4% ethanol, 4%
polyethylene glycol-600, 1% gelatin, 0.9% NaCl suspension; (3) DHEA (10
10 mg, percutaneously, once daily) in 0.5 ml of 50% ethanol, 50% propylene
glycol; and (4) both EM-800 and DHEA. Treatment was initiated 3 days
before the oral administration of DMBA. EM-800 was synthesized in the
Medicinal Chemistry Division of our laboratory while DHEA was
purchased from Steraloids Inc., Wilton, NH.

15 Many of the control animals and some of EM-800- or DHEA-treated
animals were sacrificed by cervical dislocation under isoflurane-induced
anesthesia 6 months after DMBA administration because of the too large
size of tumors. The values of tumor size and number of these rats at
sacrifice, together with those measured at later time intervals from the
20 surviving animals, were used for the later analysis of the incidence of
tumors, average tumor number per tumor-bearing rat and average tumor
size per tumor-bearing animal. The remaining animals (9 rats from
control and 13-19 rats from each other group) continued to receive
treatment for another 3-month period in order to observe long-term
25 preventive potency of DHEA and EM-800 alone or in combination. Rats
were sacrificed 279 days after DMBA administration. The uteri, vaginas,
and ovaries were immediately removed, freed from connective and
adipose tissue, and weighed.

30 Sample collection and processing

Twenty-four-hour urinary samples were collected at the end of the
experiment from the first 9 rats of each group following transfer in

5 metabolic cages (Allentown Caging Equipment Co., Allentown, NJ). Two
urinary samples were collected and analyzed on different days for each
animal in order to minimize the influence of daily variation. Therefore,
each value shown represents the mean of the two measurements
performed on two different days. 0.5 ml of toluene was added into the
10 urine collecting tubes to prevent urine evaporation and bacterial growth
and the urinary volume was recorded. Trunk blood was collected at
sacrifice and was allowed to clot at 4°C overnight before centrifugation at
3000 rpm for 30 min.

15 Analysis of urine and serum biochemical parameters

Fresh samples were used for the assay of urinary creatinine, calcium, and
phosphorus as well as serum total alkaline phosphatase (tALP) activity,
cholesterol and triglycerides. These biochemical parameters were
measured automatically with a Monarch 2000 Chemistry System
20 (Instrumentation Laboratory Co. Lexington, MA) under Good
Laboratory Practice conditions. Urinary hydroxyproline was measured
as described (Podenphant et al., Clinica Chimica Acta 142: 145-148, 1984).²⁷

Bone mass measurements

25 Rats were anesthetized with an i.p. injection of ketamine hydrochloride
and diazepam at doses of 50 and 4 mg/kg B.W., respectively. The whole
skeleton and the right femur were scanned using dual energy X-ray
absorptiometry (DEXA; QDR 2000-7.10C, Hologic, Waltham, MA)
equipped with a Regional High Resolution software. The scan field sizes
30 were 28.110 x 17.805 cm and 5.0 x 1.902 cm, the resolutions were 0.1511 x
0.0761 cm and 0.0254 x 0.0127 cm, while the scan speeds were 0.3608 and
0.0956 mm/sec for total skeleton and femur, respectively. Both bone

5 mineral content (BMC) and bone mineral density (BMD) of total skeleton, lumbar spine, and femur were measured on the scan images of total skeleton and femur.

Statistical analyses

10 Statistical significance was measured according to the multiple range test of Duncan-Kramer (Biometrics 12: 307-310, 1956). Analysis of the incidence of development of mammary tumors was performed using the Fisher's exact test (Conover, Practical nonparametric statistics, 2nd, Edition 153-170, 1980). The data are presented as means \pm S.E.M..

RESULTS

Effect on the development of DMBA-induced mammary carcinoma

As illustrated in figure 1, 95% of control animals developed palpable mammary tumors by 279 days after DMBA administration. Treatment with DHEA or EM-800 partially prevented the development of DMBA-induced mammary carcinoma and the incidence was thus reduced to 57% ($p < 0.01$) and 38% ($p < 0.01$), respectively. Interestingly, combination of the two compounds led to a significantly higher inhibitory effect than those achieved by each compound alone ($p < 0.01$ versus DHEA or EM-800 alone). In fact, the only two tumors which developed in the group of animals treated with both compounds disappeared before the end of experiment.

30 Treatment with DHEA or EM-800 decreased average tumor number per tumor-bearing animal from 4.7 ± 0.5 tumors in control animals to 3.4 ± 0.7 (N.S.) and 1.4 ± 0.3 ($p < 0.01$) tumors/animal, respectively, while no tumor was found at the end of the experiment in the animals who received both

5 drugs ($p < 0.01$ versus the three other groups) (Fig. 2A). One of the two
tumors which later disappeared was present from day 79 to day 201
following DMBA administration while the other tumor was palpable
from day 176 to day 257. It can be seen in Fig. 2B that DHEA or EM-800
10 alone decreased average tumor area per tumor-bearing animal from $12.8 \pm 1.3 \text{ cm}^2$ at the end of the experiment to $10.2 \pm 2.1 \text{ cm}^2$ (N.S.) and $7.7 \pm 1.8 \text{ cm}^2$ (N.S.), respectively, while the combination treatment resulted in a
zero value ($p < 0.01$ versus the three other groups). The two tumors which
developed in the group of animals treated with both DHEA and EM-800
did not grow larger than 1 cm^2 . It should be mentioned that the real
15 values of average tumor area as well as the average tumor number per
tumor-bearing animal in the control group should be higher than the
values presented in Figure 2, since many rats had to be sacrificed before
the end of the experiment because of the excessive size of tumors. The
values measured at time of sacrifice were thus included as such in the
20 calculations made at later time intervals in order to minimize a bias in the
control group which, in any case, remained significantly above the other
groups.

Effect on bone

25 Long-term percutaneous administration of DHEA to female rats induced
6.9% ($p < 0.01$), 10.6% ($p < 0.05$), and 8.2% ($p < 0.01$) increases in bone
mineral density (BMD) of total skeleton, lumbar spine, and femur,
respectively (Table 2). On the other hand, no significant change was
found in the animals treated with EM-800. Furthermore, when both
30 compounds were administered simultaneously, the values obtained were
comparable to those achieved with DHEA alone.

5 Treatment with DHEA increased serum total alkaline phosphatase
(tALP) activity by 74% ($p<0.05$), but had no effect on daily urinary
calcium and phosphorus excretion and on the urinary ratio of
hydroxyproline to creatinine (Table 3). On the other hand, treatment
with EM-800 decreased the urinary hydroxyproline to creatinine ratio by
10 48% ($p<0.01$), but had no statistically significant influence on daily
urinary calcium or phosphorus excretion and serum tALP activity. The
combination of DHEA and EM-800 led to an increase in serum tALP
activity ($p<0.01$) similar to that achieved with DHEA alone and reduced
the urinary hydroxyproline to creatinine ratio by 69%, a value
15 significantly ($p<0.01$) lower than that achieved with EM-800 alone. In
addition, the combination of the two drugs significantly reduced daily
urinary calcium and phosphorus excretion by 84% ($p<0.01$) and 56%
($p<0.01$), respectively, while no significant change was observed with
each drug alone (Table 3).

Effect on serum lipid levels

Long-term treatment with EM-800 lowered serum triglyceride and
cholesterol levels by 72% ($p<0.01$) and by 45% ($p<0.01$), respectively,
whereas long-term administration of DHEA decreased serum
25 triglycerides levels by 60% ($p<0.01$), serum cholesterol levels being
unaffected. Moreover, 42% ($p<0.01$) and 52% ($p<0.01$) decreases in serum
triglyceride and cholesterol concentrations were measured in the animals
treated with both EM-800 and DHEA (Fig. 3).

5 Table 2. Effect of treatment with DHEA (10 mg, percutaneously, once daily) or EM-800 (75 µg, orally, once daily) alone or in combination for 9 months on bone mineral density (BMD) of femur, lumbar spine, and total skeleton in the female rat. Measurements were performed in 9 rats per group. *: p<0.05; **: p<0.01, experimental versus control.

10

| GROUP | BMD (g/cm ²) | | |
|----------------|--------------------------|------------------|-------------------|
| | TOTAL SKELETON | LUMBAR SPINE | FEMUR |
| CONTROL | 0.1371 ± 0.0025 | 0.1956 ± 0.0067 | 0.3151 ± 0.0063 |
| DHEA (10 mg) | 0.1465 ± 0.0010** | 0.2163 ± 0.0049* | 0.3408 ± 0.0038** |
| EM-800 (75 µg) | 0.1356 ± 0.0017 | 0.1888 ± 0.0045 | 0.3097 ± 0.0047 |
| DHEA + EM-800 | 0.1498 ± 0.0019** | 0.2108 ± 0.0061 | 0.3412 ± 0.0056** |

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Table 3. Effect of treatment with DHEA (10 mg, percutaneously, once daily) or EM-800 (75 µg, orally, once daily) alone or in combination for 9 months on parameters of bone metabolism in the rat: daily urinary calcium and phosphorus excretion, urinary hydroxyproline to creatinine ratio (HP/Cr), and serum total alkaline phosphatase activity (tALP). Samples were obtained from 9 animals per group. *: $p < 0.05$; **: $p < 0.01$ experimental versus control.

| GROUP | URINE | | | SERUM |
|----------------|----------------------------|-------------------------------|----------------------|------------------|
| | CALCIUM (µmol/24h/100g) | PHOSPHORUS (µmol/24h/100g) | HP/Cr (µmol/mmol) | tALP (IU/L) |
| CONTROL | 23.17 ± 1.55 | 132.72 ± 6.08 | 13.04 ± 2.19 | 114.25 ± 14.04 |
| DHEA (10 mg) | 25.87 ± 3.54 | 151.41 ± 14.57 | 14.02 ± 1.59 | 198.38 ± 30.76* |
| EM-800 (75 µg) | 17.44 ± 4.5 | 102.03 ± 25.13 | 6.81 ± 0.84** | 114.11 ± 11.26 |
| DHEA + EM-800 | 3.71 ± 0.75** | 59.06 ± 4.76** | 4.06 ± 0.28** | 204.38 ± 14.20** |

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Example 2

ABSTRACT

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In the mammary gland, androgens are formed from the precursor steroid dehydroepiandrosterone (DHEA). Clinical evidence indicates that androgens have inhibitory effects on breast cancer. Estrogens, on the other hand, stimulate the development and growth of breast cancer. We studied the effect of DHEA alone or in combination with the newly described pure antiestrogen, EM-800, on the growth of tumor xenografts formed by the human breast cancer cell line ZR-75-1 in ovariectomized nude mice.

15

Mice received daily subcutaneous injections of 0.5 μ g estrone (an estrogenic hormone) immediately after ovariectomy. EM-800 (15, 50 or 100 μ g) was given orally once daily. DHEA was applied twice daily (total dose 0.3, 1.0 or 3.0 mg) to the dorsal skin either alone or in combination with a 15 μ g daily oral dose of EM-800. Changes in tumor size in response to the treatments were assessed periodically in relation to the measurements made on the first day. At the end of the experiments, tumors were dissected and weighed.

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A 9.4-fold increase in tumor size in 9.5 months was observed in ovariectomized mice receiving estrone alone in comparison with mice not receiving estrone. Administration of 15, 50 or 100 μ g EM-800 in estrone-supplemented ovariectomized led to inhibitions of 88%, 93%, and 94% in tumor size, respectively. DHEA, on the other hand, at doses of 0.3, 1.0 or 3.0 mg inhibited terminal tumor weight by 67%, 82%, and 85%, respectively. Comparable inhibitions in tumor size were obtained with a

30

5 daily 15 µg oral dose of EM-800 with or without different doses of percutaneous DHEA.

DHEA and EM-800 independently suppressed the growth of estrone-stimulated ZR-75-1 mouse xenograft tumors in nude mice.
10 Administration of DHEA at the defined doses does not alter the inhibitory effect of EM-800.

MATERIALS AND METHODS

ZR-75-1 cells

15 ZR-75-1 human breast cancer cells were obtained from the American Type Culture Collection (Rockville, MD) and routinely cultured as monolayers in RPMI 1640 medium supplemented with 2 mM L-glutamine, 1 mM sodium pyruvate, 100 IU penicillin/ml, 100 µg streptomycin/ml, and 10% fetal bovine serum, under a humidified
20 atmosphere of 95% air/5% CO₂ at 37°C as described (Poulin and Labrie, Cancer Res. 46: 4933-4937, 1986; Poulin et al., Breast Cancer Res. Treat. 12: 213-225, 1988). Cells were passaged weekly after treatment with 0.05% trypsin:0.02% EDTA (w/v). The cell cultures used for the experiments described in this report were derived from passage 93 of the cell line ZR-
25 75-1.

Animals

Female homozygous Harlan Sprague-Dawley (nu/nu) athymic mice (28- to 42-day-old) were obtained from HSD (Indianapolis, Indiana, USA).
30 Mice were housed in vinyl cages with air filter tops in laminar air flow hoods and maintained under pathogen-limited conditions. Cages,

5 bedding, and food were autoclaved before use. Water was autoclaved,
acidified to pH 2.8, and provided ad libitum.

Cell inoculation

10 Mice were bilaterally ovariectomized (OVX) one week before tumor cell
inoculation under anesthesia achieved by intraperitoneal injection of 0.25
ml/animal of Avertin (amylic alcohol: 0.8 g/100 ml 0.9% NaCl; and
tribromo ethanol: 2g/100 ml 0.9% NaCl). 1.5×10^6 ZR-75-1 cells in
logarithmic growth phase were harvested after the treatment of
15 monolayer with 0.05% trypsin/0.02% EDTA (w/v), were suspended in
0.1 ml of culture medium containing 25% Matrigel and were inoculated
subcutaneously on both flanks of the animals using a 1 inch-long 20-
gauge needle as described previously (Dauvois et al., Cancer Res. 51:
3131-3135, 1991). In order to facilitate growth of the tumors, each animal
received daily subcutaneous injection of 10 μ g of estradiol (E_2) in vehicle
20 composed of 0.9% NaCl 5% ethanol 1% gelatin for 5 weeks. After
appearance of palpable ZR-75-1 tumors, tumor diameter was measured
with calipers and mice having tumor diameter between 0.2 and 0.7 cm
were selected for this study.

25 Hormonal treatment

All animals, except those in the control OVX group, received daily
subcutaneous injections of 0.5 μ g estrone (E_1) in 0.2 ml of 0.9% NaCl 5%
ethanol 1% gelatin. In the indicated groups, DHEA was administered
percutaneously twice daily at the doses of 0.3, 1.0 or 3.0 mg/animal
30 applied in a volume of 0.02 ml on the dorsal skin area outside the area of
tumor growth. DHEA was dissolved in 50% ethanol 50% propylene
glycol. EM-800, ((+)-7-pivaloyloxy-3-(4'-pivaloyloxyphenyl)-4-methyl-2-

5 (4''-(2'''-piperidinoethoxy)phenyl)-2H-benzopyran), was synthesized as
described earlier (Gauthier et al., J. Med. Chem. 40: 2117-2122, 1997) in
the medicinal chemistry division of the Laboratory of Molecular
Endocrinology of the CHUL Research Center. EM-800 was dissolved in
10 4% (v/v) ethanol 4% (v/v) polyethylene glycol (PEG) 600 1% (w/v)
gelatin 0.9% (w/v) NaCl. Animals of the indicated groups received daily
oral doses of 15 µg, 50 µg, or 100 µg of EM-800 alone or in combination
with DHEA while animals of the OVX group received the vehicle (0.2 ml
4% ethanol 4% PEG 600 1% gelatin 0.9% NaCl) alone. Tumors were
measured once a week with Vernier calipers. Two perpendicular
15 diameters in cms (L and W) were recorded and tumor area (cm²) was
calculated using the formula: $L/2 \times W/2 \times \pi$ (Dauvois et al., Cancer Res.,
51: 3131-3135, 1991). The area measured on the first day of treatment was
taken as 100% and changes in tumor size were expressed as percentage of
initial tumor area. In case of subcutaneous tumors in general, it is not
20 possible to accurately access three dimensional volume of tumor,
therefore, only tumors areas were measured. After 291 days (or 9.5
months) of treatment, the animals were sacrificed.

The categories of responses were evaluated as described (Dauvois et al.,
25 Breast Cancer Res. Treat. 14: 299-306, 1989; Dauvois et al., Eur. J. Cancer
Clin. Oncol. 25: 891-897, 1989; Labrie et al., Breast Cancer Res. Treat. 33:
237-244, 1995). In short, partial regression corresponds to the tumors that
regressed equal to or more than 50% of their original size; stable response
refers to tumors that regressed less than 50% of the original size or
30 progressed less than 50% of their original size, while complete regression
refers to those tumors that were undetectable at the end of treatment.
Progression refers to tumors that progressed more than 50% compared

5 with their original size. At the end of the experiment, all animals were killed by decapitation. Tumors, uterus, and vagina were immediately removed, freed from connective and adipose tissues, and weighed.

Statistical analysis

10 Statistical significance of the effects of treatments on tumor size was assessed using an analysis of variance (ANOVA) evaluating the effects due to DHEA, EM-800, and time, and repeated measures in the same animals performed at the initiation and at the end of the treatment (subjects within group factor). The repeated measures at time 0 and after
15 9.5 months of treatment constitute randomized blocks of animals. The time is thus analyzed as a within-block effect while both treatments are assessed as between-block effects. All interactions between main effects were included in the model. The significance of the treatment factors and of their interactions was analyzed using the subjects within group as the
20 error term. Data were log-transformed. The hypotheses underlying the ANOVA assumed the normality of the residuals and the homogeneity of variance.

25 A posteriori pairwise comparisons were performed using Fisher's test for least significant difference. Main effects and the interaction of treatments on body weight and organ weight were analyzed using a standard two-way ANOVA with interactions. All ANOVAs were performed using SAS program (SAS Institute, Cary, NC, USA). Significance of differences were declared using a 2-tailed test with an overall level of 5%.

30 Categorical data were analyzed with a Kruskal-Wallis test for ordered categorical response variables (complete response, partial response,

stable response, and progression of tumor). After overall assessment of a treatment effects, subsets of the results presented in Table 4 were analyzed adjusting the critical p-value for multiple comparisons. The exact p-values were calculated using StatXact program (Cytel, Cambridge, MA, USA).

Data are expressed as means \pm standard error of the mean (SEM) of 12 to 15 mice in each group.

RESULTS

As illustrated in Fig. 4A, human ZR-75-1 tumors increased by 9.4-fold over 291 days (9.5 months) in ovariectomized nude mice treated with a daily 0.5 μ g subcutaneously administered dose of estrone while in control OVX mice who received the vehicle alone, tumor size was decreased to 36.9% of the initial value during the course of the study.

Treatment with increasing doses of percutaneous DHEA caused a progressive inhibition of E₁-stimulated ZR-75-1 tumor growth. Inhibitions of 50.4%, 76.8%, and 80.0% were achieved at 9.5 months of treatment with the 0.3 mg, 1.0 mg, and 3.0 mg daily doses per animal of DHEA, respectively (Fig. 4A). In agreement with the decrease in total tumor load, treatment with DHEA led to a marked decrease of the average weight of the tumors remaining at the end of the experiment. In fact, average tumor weight decreased from 1.12 ± 0.26 g in control E₁-supplemented ovariectomized nude mice to 0.37 ± 0.12 g ($P = .005$), 0.20 ± 0.06 g ($P = .001$), and 0.17 ± 0.06 g ($P = .0009$) in the groups of animals receiving the daily 0.3, 1.0 and 3.0 mg doses of DHEA, respectively (Fig. 4B).

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At the daily doses of 15 μ g, 50 μ g, and 100 μ g, the antiestrogen EM-800 inhibited estrogen-stimulated tumor size by 87.5% ($P<.0001$), 93.5% ($P<.0001$), and 94.0% ($P=.0003$), respectively (Fig. 5A) when compared to the tumor size in control animals at 9.5 months. The tumor size reductions achieved with the three EM-800 doses are not significantly different between each other. As illustrated in Fig. 4B, tumor weight at the end of the 9.5-month study was decreased from 1.12 ± 0.26 g in control E_1 -supplemented OVX mice to 0.08 ± 0.03 g, 0.03 ± 0.01 g and 0.04 ± 0.03 g in animals treated with the daily 15 μ g, 50 μ g, and 100 μ g doses of EM-800, respectively ($P<.0001$ at all doses of EM-800 vs E_1 supplemented OVX).

20

25

As mentioned above, the antiestrogen EM-800, at the daily oral dose of 15 μ g, caused a 87.5% inhibition of estrone-stimulated tumor growth measured at 9.5 months. The addition of DHEA at the three doses used had no significant effect on the already marked inhibition of tumor size achieved with the 15 μ g daily dose of the antiestrogen EM-800 (Fig. 5B). Thus, average tumor weight was dramatically reduced from 1.12 ± 0.26 g in control estrone-supplemented mice to 0.08 ± 0.03 g ($P<.0001$), 0.11 ± 0.04 g ($P=.0002$), 0.13 ± 0.07 g ($P=.0004$) and 0.08 ± 0.05 g ($P<.0001$) in the animals who received the daily dose of 15 μ g of the antiestrogen alone or in combination with the 0.3, 1.0, and 3.0 mg doses of DHEA, respectively (no significant difference was noted between the 4 groups) (Fig. 4B).

30

It was also of interest to examine the categories of responses achieved with the above-indicated treatments. Thus, treatment with the increasing doses of DHEA decreased, although not to a level of statistical

5 significance ($P=0.088$), the number of progressing tumors from 87.5% in
the control OVX animals supplemented with estrone to values of 50.0%,
53.3%, and 66.7% in the animals treated with the daily doses of 0.3, 1.0 or
3.0 mg of DHEA (Table 4). Complete responses, on the other hand,
increased from 0% in the estrone-supplemented mice to 28.6%, 26.7%,
10 and 20.0% in the animals receiving the 0.3, 1.0, and 3.0 mg daily doses of
percutaneous DHEA. Stable responses, on the other hand, were
measured at 12.5%, 21.4%, 20.0%, and 13.3% in the control E_1 -
supplemented mice and in the three groups of animals who received the
above-indicated doses of DHEA, respectively. In control ovariectomized
15 mice, the rates of complete, partial and stable responses were measured
at 68.8%, 6.2%, and 18.8%, respectively, while progression was seen in
only 6.2% of tumors (Table 4).

Complete responses or disappearance of the tumors were achieved in
20 29.4%, 33.3%, 26.7%, and 35.3% of tumors in the animals who received the
antiestrogen EM-800 ($P=0.0006$) alone (15 μ g) or in combination with the
0.3 mg, 1.0 mg, or 3.0 mg of DHEA, respectively (Table 4). Progression,
on the other hand, was seen in 35.3%, 44.4%, 53.3%, and 17.6% of the
tumors, in the same groups of animals, respectively. There is no
25 significant difference between the groups treated with EM-800, either
alone or in combination with DHEA.

No significant effect of DHEA or EM-800 treatment was observed on
body weight adjusted for tumor weight. Treatment of OVX mice with
30 estrone, increased uterine weight from 28 ± 5 mg in OVX control mice to
 132 ± 8 mg ($P<0.01$) while increasing doses of DHEA caused a progressive
but relatively small inhibition of the stimulatory effect of estrone which

5 reached 26% ($P=.0008$) at the highest dose of DHEA used. It can be seen
in the same figure that estrone-stimulated uterine weight was decreased
from 132 ± 8 mg in control estrone-supplemented mice to 49 ± 3 mg, $36 \pm$
2 mg, and 32 ± 1 mg ($P<.0001$ at all doses vs control) with the daily oral
doses of $15\mu\text{g}$, $50\mu\text{g}$, or $100\mu\text{g}$ of EM-800 (overall $P<.0001$), respectively.
10 Fifteen micrograms ($15\mu\text{g}$) EM-800 in combination with the 0.3 mg, 1.0
mg or 3.0 mg daily doses of DHEA, uterine weight was measured at $46 \pm$
3 mg, 59 ± 5 mg and 69 ± 3 mg, respectively.

On the other hand, treatment with estrone increased vaginal weight from
15 14 ± 2 mg in OVX animals to 31 ± 2 mg ($P<.01$) while the addition of
DHEA had no significant effect. Vaginal weight was then reduced to 23
 ± 1 mg, 15 ± 1 mg, and 11 ± 1 mg following treatment with the daily 15
 μg , $50\mu\text{g}$ or $100\mu\text{g}$ doses of EM-800, respectively (overall p and pairwise
 $P<.0001$ at all doses vs control). In combination with the 0.3 mg, 1.0 mg
20 or 3.0 mg doses of DHEA and of EM-800, vaginal weight was measured
at 22 ± 1 mg, 25 ± 2 mg and 23 ± 1 mg, respectively (N.S. for all groups
versus $15\mu\text{g}$ EM-800). It should be mentioned that at the highest dose
used, namely $100\mu\text{g}$ daily, EM-800 decreased uterine weight in estrone-
supplemented OVX animals to a value not different from that of OVX
25 controls while vaginal weight was reduced to a value below that
measured in OVX controls ($P<.05$). DHEA, probably due to its
androgenic effects, partially counteracted the effect of EM-800 on uterine
and vaginal weight.

5 Table 4. Effect of percutaneous administration of DHEA or oral administration of EM-800 alone or in combination for 9.5 months on the responses (complete, partial, stable, and progression) of human ZR-75-1 breast tumor xenografts in nude mice.

| GROUP | TOTAL NUMBER OF ANIMALS | CATEGORY OF RESPONSE | | | |
|--------------------------------------|-------------------------------|----------------------|----------|----------|-------------|
| | | Complete | Partial | Stable | Progression |
| | | Number and (%) | | | |
| OVX | 16 | 11 (68.8) | 1 (6.2) | 3 (18.8) | 1 (6.2) |
| OVX + E1 (0.5 µg) | 16 | 0 (0) | 0 (0) | 2 (12.5) | 14 (87.5) |
| OVX + E1 (0.5 µg) + DHEA | 0.3 mg | 4 (28.6) | 0 (0) | 3 (21.4) | 7 (50.0) |
| | 1.0 mg | 4 (26.7) | 0 (0) | 3 (20.0) | 8 (53.3) |
| | 3.0 mg | 3 (20.0) | 0 (0) | 2 (13.3) | 10 (66.7) |
| OVX + E1 (0.5 µg) + EM-800 | 15 µg | 5 (29.4) | 1 (5.9) | 5 (29.4) | 6 (35.3) |
| | 50 µg | 4 (25.0) | 3 (18.8) | 5 (31.2) | 4 (25.0) |
| | 100 µg | 8 (50.0) | 0 (0) | 3 (18.8) | 5 (31.2) |
| OVX + E1 (0.5 µg) + EM-800 + DHEA | 0.3 mg | 6 (33.3) | 0 (0) | 4 (22.2) | 8 (44.4) |
| | 1.0 mg | 4 (26.7) | 0 (0) | 3 (20.0) | 8 (53.3) |
| | 3.0 mg | 6 (35.3) | 0 (0) | 8 (47.1) | 3 (17.6) |

E₁ = Estrone; DHEA = dehydroepiandrosterone; OVX = ovariectomized

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Example 3

**Effect of the preferred compound of the invention on
cholesterol levels of female ovariectomized rats**

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Animals and treatment

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Fifty to 60 day-old female Sprague-Dawley rats (CrI:CD(SD)Br) (Charles River Laboratory, St-Constant, Canada) weighing approximately 190 g at the time of ovariectomy were used. The animals were acclimated to the environmental conditions (temperature: $22 \pm 3^{\circ}\text{C}$; humidity: $50 \pm 20\%$; 12-h light-12-h dark cycles, lights on at 07:15h) for 1 week before the surgery. The animals were housed three per cage and were allowed free access to tap water and a pelleted certified rodent feed (Lab Diet 5002, Ralston Purina, St-Louis, MO). The experiment was conducted in a Canadian Council on Animal Care approved facility in accordance with the CCAC Guide for Care and Use of Experimental Animals.

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One hundred thirty-six female rats were ovariectomized under Isoflurane anesthesia on day 0 of the study and were randomly distributed into 17 groups of animals to conduct the study outlined below:

5

Group 1: OVX CONT

Group 2: OVX + EM-800 (0.01 mg/kg, po, ID)

Group 3: OVX + EM-800 (0.03 mg/kg, po, ID)

Group 4: OVX + EM-800 (0.1 mg/kg, po, ID)

Group 5: OVX + EM-800 (0.3 mg/kg, po, ID)

Group 6: OVX + EM-800 (1 mg/kg, po, ID)

Group 7: OVX + EM-01538 (0.01 mg/kg, po, ID)

Group 8: OVX + EM-01538 (0.03 mg/kg, po, ID)

Group 9: OVX + EM-01538 (0.1 mg/kg, po, ID)

Group 10: OVX + EM-01538 (0.3 mg/kg, po, ID)

Group 11: OVX + EM-01538 (1 mg/kg, po, ID)

Group 12: OVX + Raloxifene (EM-1105) (0.01 mg/kg, po, ID)

Group 13: OVX + Raloxifene (EM-1105) (0.03 mg/kg, po, ID)

Group 14: OVX + Raloxifene (EM-1105) (0.1 mg/kg, po, ID)

Group 15: OVX + Raloxifene (EM-1105) (0.3 mg/kg, po, ID)

Group 16: OVX + Raloxifene (EM-1105) (1 mg/kg, po, ID)

Group 17: INT CONT

5 The administration of treatments were started on day 10 of the study and
were given by oral gavage once daily until day 13 of the study. Dosing
suspensions were prepared in 0.4% methylcellulose and the
concentration was adjusted according to the mean body weight of the
group recorded on day 10 of the study in order to give 0.5 ml of dosing
10 suspension per rat. Approximately 24 hours after the last dosing,
overnight faster animals were killed by exsanguination at the abdominal
aorta under isoflurane anesthesia and blood samples were processed for
serum preparation. The uteri were removed, stripped of remaining fat
and weighed.

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Serum cholesterol and triglyceride assays

Total serum cholesterol and triglyceride levels were determined using
the Boehringer Mannheim Diagnostic Laboratory Systems).

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Example 4

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Androstene-3 β ,17 β -diol (5-diol) possesses intrinsic estrogenic activity. In addition, as a precursor sex steroid, it can be transformed into active androgens and/or other estrogens in peripheral intracrine tissues. In order to assess the relative importance of the androgenic and estrogenic components of 5-diol action on bone mass, twenty-one week old rats were ovariectomized and treated percutaneously once daily with 2, 5, or 12.5 mg of 5-diol alone or in combination with the antiandrogen Flutamide (FLU, 10 mg, s.c., once daily), and/or the antiestrogen EM-800 (100 μ g, s.c., once daily) for 12 months. Bone mineral density (BMD) was measured after 11 months of treatment. Ovariectomy (OVX) led to a 12.8% decrease in femoral BMD ($p < 0.01$) while treatment with the highest dose of 5-diol restored 34.3% of femoral BMD lost during the 11 months following OVX ($p < 0.01$). Simultaneous administration of FLU completely prevented the stimulatory effect of 5-diol on femoral BMD while the addition of EM-800 resulted in an additional 28.4% stimulation compared to the effect of 5-diol alone. The simultaneous administration of 5-diol, FLU, and EM-800 only displayed the effect of EM-800 (27%) since the effect of 5-diol was completely blocked by FLU. Comparable results were obtained on BMD of lumbar spine although lumbar spine BMD in OVX rats receiving 12.5 mg 5-diol alone, 12.5 mg 5-diol + EM-800 or 5-diol + FLU + EM-800 was restored to values not significantly different from those of intact animals. The histomorphometric analysis shows that the stimulatory effects of 5-diol on bone volume, trabecular number and the inhibitory effect on trabecular separation of secondary spongiosa of the proximal tibia metaphyseal area are abolished by FLU, but further enhanced by EM-800. The marked stimulation of serum

5 alkaline phosphatase activity obtained following the treatment with 5-
diol is 57% ($p < 0.01$ vs 12.5 mg 5-diol alone) reversed by the simultaneous
administration of FLU. Treatment with 5-diol had no statistically
significant inhibitory effect on the urinary ratio of calcium to creatinine.
10 The highest dose of 5-diol caused a significant 23% ($p < 0.01$) reduction of
serum cholesterol while the addition of EM-800 decreased serum
cholesterol by 62% ($p < 0.01$). The present data clearly show the
stimulatory effect of 5-diol on bone formation and suggest that although
5-diol is a weak estrogen, its stimulatory effect on bone formation is
predominantly mediated by an androgenic effect. Moreover, the
15 additive stimulatory effects of EM-800 and 5-diol on bone mass
demonstrate the bone-sparing effect of the anti-estrogen EM-800 in the
rat. The cholesterol-lowering activity of both 5-diol and EM-800 could
have interesting utility for the prevention of cardiovascular diseases.

Example 5

| Group | Serum Alkaline phosphatase | Urinary OH- proline /creatinin | LH ng/ml | GnRH mRNA levels silver grains per cell | Cholesterol mmol/L | Trigly- cerides mmol/L |
|---------------------|----------------------------------|--------------------------------------|---------------|--|-----------------------|------------------------------|
| | IU/L | μmol/nmol | | | | |
| Intact Control | 30 ± 3** | 15.4 ± 1.3 | 0.09 ± 0.03** | 33.7 ± 0.7** | 2.28 ± 0.12 | 1.4 ± 0.2 |
| OVX Control | 51 ± 4 | 11.7 ± 1.2 | 3.55 ± 0.50 | 44.0 ± 0.9 | 2.29 ± 0.16 | 1.1 ± 0.1 |
| OVX + MPA | 57 ± 4 | 11.7 ± 1.2 | 2.51 ± 0.20* | 35.5 ± 0.7** | 2.55 ± 0.14 | 1.3 ± 0.1 |
| OVX + E2 | 41 ± 5 | 9.2 ± 0.9 | 2.37 ± 0.45* | 40.4 ± 0.8** | 2.02 ± 0.15 | 0.8 ± 0.1 |
| OVX + DHT | 56 ± 5 | 7.8 ± 0.7* | 1.55 ± 0.27** | 38.8 ± 0.7** | 2.44 ± 0.16 | 0.9 ± 0.1 |
| OVX + DHEA | 201 ± 25** | 7.3 ± 1.0* | 0.02 ± 0.01** | 34.5 ± 0.7** | 1.78 ± 0.16* | 0.8 ± 0.1 |
| OVX + DHEA + FLU | 103 ± 10** | 14.5 ± 1.2 | 1.13 ± 0.24** | 41.5 ± 0.7* | 2.27 ± 0.15 | 0.8 ± 0.1 |
| OVX + DHEA + EM-800 | 202 ± 17** | 6.4 ± 1.0** | LD** | 39.2 ± 0.7** | 0.63 ± 0.09** | 1.0 ± 0.2 |

LD: Limit of Detection : 0.01 ng/ml

* p<0.05; ** p<0.01 versus OVX Control

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Synthesis of (S)-(+)-7-hydroxy-3-(4'-hydroxyphenyl)-4-methyl-2-(4''-(2'''-piperidinoethoxy)phenyl)-2H-1-benzopyran hydrochloride EM-01538
(EM-652, HCl)

The reaction scheme illustrates the multi-step synthesis of EM-01538:

- Starting Materials:** 1 (Resorcinol) and 2 (3-(4-hydroxyphenyl)-2-hydroxypropanoic acid).
- Step A:** Condensation of 1 and 2 to form intermediate 3.
- Step C:** Protection of one hydroxyl group in 3 as a tetrahydropyranyl ether (THPE) to yield 4.
- Step D:** Reaction of 4 with 5 (4-(benzyloxymethyl)piperidine) to form 6.
- Step E:** Coupling of 6 with another molecule of 2 to form 7.
- Step F:** Cyclization of 7 to form the chromane core 8.
- Steps G, H:** Functionalization of 8 with a chiral auxiliary (1S,2S)-1-((tert-butylideneamino)oxy)-2-methylpropyl ester to form 9.
- Step I:** Deprotection of the THPE group in 9 to yield 10.
- Step J:** Final functionalization of 10 to produce the target compound EM-01538.

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Step A: $\text{BF}_3 \cdot \text{Et}_2\text{O}$, toluene; 100°C ; 1 hour.

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Step C: 3,4-dihydropyran, p-toluenesulfonic acid monohydrate, ethyl acetate; 25°C under nitrogen, 16 hours, and then crystallization in isopropanol.

15

Steps D, E, and F:

- (1) piperidine, toluene, Dean & Stark apparatus, reflux under nitrogen;
- (2) 1,8-diazabicyclo[5, 4, 0]undec-7-ene, DMF, reflux 3 hours;
- (3) CH_3MgCl , THF, -20 to 0°C and then room temperature for 24 hours;

20

Steps G, H: (1S)-(+)-10-camphorsulfonic acid, acetone, water, toluene, room temperature, 48 hours.

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Step HH: 95% ethanol, 70°C , then room temperature 3 days.

Step HHR: Recycling of mother liquor and wash of step HH (S)-10-camphorsulfonic acid, reflux; 36 hours, then room temperature for 16 hours.

Step I:

- (1) DMF aq., Na_2CO_3 , ethyl acetate;
- (2) ethanol, dilute HCl;
- (3) water.

5 **Synthesis of 2-tetrahydropyranyloxy-4-hydroxy-2'-(4''-**
 tetrahydropyranyloxyphenyl) acetophenone (4). A suspension of 2,4-
 dihydroxy-2'-(4''-hydroxyphenyl)acetophenone **3** (97.6 g, 0.4 mole)
 (available from Chemsyn Science Laboratories, Lenexa, Kansas) in 3,4-
10 dihydropyran (218 ml, 3.39 mole) and ethyl acetate (520 ml) was treated
 with p-toluenesulfonic acid monohydrate (0.03 g, 0.158 mmole) at about
 25°C. The reaction mixture was stirred under nitrogen with no external
 heating for about 16 hours. The mixture was then washed with a
 solution of sodium bicarbonate (1 g) and sodium chloride (5 g) in water
 (100 ml). The phases were separated and the organic phase was washed
15 with brine (20 ml). Each wash was back extracted with 50 ml ethyl
 acetate. All the organic phases were combined and filtered through
 sodium sulfate.

 Solvent (about 600 ml) was removed by distillation at atmospheric
20 pressure and isopropanol (250 ml) was added. Additional solvent (about
 300 ml) was distilled at atmospheric pressure and isopropanol (250 ml)
 was added. Additional solvent (about 275 ml) was distilled at
 atmospheric pressure and isopropanol (250 ml) was added. The solution
 was cooled at about 25°C with stirring and after about 12 hours, the
25 crystalline solid was filtered, washed with isopropanol and dried (116.5
 g, 70%).

Synthesis of 4-hydroxy-4-methyl-2-(4'-[2''-piperidino]-ethoxy)phenyl-3-
 (4'''-tetrahydropyranyloxy)phenyl-7-tetrahydropyranyloxy-chromane
30 **(10).** A solution of 2-tetrahydropyranyloxy-4-hydroxy-2'-(4''-
 tetrahydropyranyloxyphenyl)acetophenone **4** (1 kg, 2.42 mole), 4-[2-(1-
 piperidino)ethoxy]benzaldehyde **5** (594 g, 2.55 mole) (available from

5 Chemsyn Science Laboratories, Lenexa, Kansas) and piperidine (82.4 g, 0.97 mole) (available from Aldrich Chemical Company Inc., Milwaukee, Wis.) in toluene (8L) was refluxed under nitrogen with a Dean & Stark apparatus until one equivalent of water (44 mL) was collected.

10 Toluene (6.5 L) was removed from the solution by distillation at atmospheric pressure. Dimethylformamide (6.5 L) and 1,8-diazabicyclo[5,4,0]undec-7-ene (110.5 g, 0.726 mole) were added. The solution was agitated for about 8 hours at room temperature to isomerize the chalcone 8 to chromanone 9 and then added to a mixture of water and ice (8 L) and toluene (4 L). The phases were separated and the
15 toluene layer washed with water (5 L). The combined aqueous washes were extracted with toluene (3 x 4 L). The combined toluene extracts were finally washed with brine (3 x 4 L), concentrated at atmospheric pressure to 5.5 L and then cooled to -10°C.

20 With continued external cooling and stirring under nitrogen, a 3M solution of methylmagnesium chloride in THF (2.5 L, 7.5 mole) (available from Aldrich Chemical Company Inc., Milwaukee, Wis.) was added, maintaining the temperature below 0°C. After all the Grignard reagent
25 was added, the external cooling was removed and the mixture allowed warm to room temperature. The mixture was stirred at this temperature for about 24 hours.

30 The mixture was again cooled to about -20°C and with continued external cooling and stirring, saturated ammonium chloride solution (200 ml) was added slowly, maintaining the temperature below 20°C. The mixture was stirred for 2 hours and then added the saturated ammonium

5 chloride solution (2L) and toluene (4 L) and agitated for five minutes.
The phases were separated and the aqueous layer extracted with toluene
(2 x 4L). The combined toluene extracts were washed with dilute
hydrochloric acid until the solution became homogenous and then with
brine (3 x 4 L). The toluene solution was finally concentrated at
10 atmospheric pressure to 2L. This solution was used directly in the next
step.

Synthesis of (2R,S)-7-hydroxy-3-(4'-hydroxyphenyl)-4-methyl-2-(4''-[2'''-
piperidino]ethoxy)phenyl)-2H-1-benzopyran (1S)-10-
15 camphorsulphonic acid salt (± 12). To the toluene solution of 4-hydroxy-
4-methyl-2-(4'-[2''-piperidino]-ethoxy)-phenyl-3-(4'''-
tetrahydropyranyloxy)phenyl-7-tetrahydropyranyloxychromane (10)
was added acetone (6 L), water (0.3 L) and (S)-10-camphorsulphonic acid
(561 g, 2.42 mole) (available from Aldrich Chemical Company Inc.,
20 Milwaukee, Wis.). The mixture was agitated under nitrogen for 48 hours
after which time the solid (2R,S)-7-hydroxy-3-(4'-hydroxyphenyl)-4-
methyl-2-(4''-[2'''-piperidino]ethoxy)phenyl)-2H-1-benzopyran (1S)-10-
camphorsulphonic acid salt (12) was filtered, washed with acetone and
dried (883 g). This material was used in the next (HH) step without
25 further purification.

Synthesis of (2S)-7-hydroxy-3-(4'-hydroxyphenyl)-4-methyl-2-(4''-[2'''-
piperidino]ethoxy)phenyl)-2H-1-benzopyran (1S)-10-
camphorsulphonic acid salt (13, (+)-EM-652(1S)-CSA salt). A
30 suspension of (2R,S)-7-hydroxy-3-(4'-hydroxyphenyl)-4-methyl-2-(4''-[2'''-
piperidino]ethoxy)phenyl)-2H-benzopyran (1S)-10-camphorsulphonic
acid salt ± 12 (759 g) in 95% ethanol was heated with stirring to about

70°C until the solid had dissolved. The solution was allowed to cool to room temperature with stirring then seeded with a few crystals of (2S)-7-hydroxy-3-(4'-hydroxyphenyl)-4-methyl-2-(4''-[2'''-piperidino]ethoxy)phenyl)-2H-1-benzopyran (1S)-10-camphorsulphonic acid salt 13. The solution was stirred at room temperature for about three days in total. The crystals were filtered, washed with 95% ethanol and dried (291 g, 76%). The de of the product was 94.2% and the purity 98.8%.

Synthesis of (S)-(+)-7-hydroxy-3-(4'-hydroxyphenyl)-4-methyl-2-(4''-(2'''-piperidinoethoxy)phenyl)-2H-1-benzopyran hydrochloride EM-01538 (EM-652, HCl). A suspension of compound 13 (EM-652-(+)-CSA salt, 500 mg, 0.726 mmol) in dimethylformamide (11 µL, 0.15 mmol) was treated with an 0.5 M aqueous sodium carbonate solution (7.0 mL, 3.6 mmol), and stirred for 15 min. The suspension was treated with ethyl acetate (7.0 mL) and stirred during 4 h. The organic phase was then washed with an aqueous saturated sodium carbonate solution (2 x 5 mL) and brine (1 x 5 mL) dried over magnesium sulfate, and concentrated. A solution of the resulting pink foam (EM-652) in ethanol (2 mL) was treated with 2 N hydrochloric acid (400 µL, 0.80 mmol), stirred for 1 h, treated with distilled water (5 mL), and stirred during 30 min. The resulting suspension was filtered, washed with distilled water (5 mL), dried in air and under high vacuum (65°C) to give a creamy powder (276 mg, 77%) : Fine off-white powder ; Scanning Calorimetry: Melting peak onset at 219°C, $\Delta H = 83 \text{ J/g}$; $[\alpha]^{24}_{\text{D}} = 154^\circ$ in methanol 10 mg/ml. ;¹H NMR (300 MHz, CD₃OD) δ (ppm) 1.6 (broad, 2H, H-4'''), 1.85 (broad, 4H, H-3''' and 5'''), 2.03 (s, 3H, CH₃), 3.0 and 3.45 (broad, 4H, H-2''' and 6'''), 3.47 (t, J=4.9Hz, 2H, H-3'''), 4.26 (t, J=4.9Hz, 2H, H-2'''), 5.82 (s, 1H,

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PHARMACEUTICAL COMPOSITION EXAMPLES

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Set forth below, by way of example and not of limitation, are several pharmaceutical compositions utilizing preferred active SERM EM-800 or EM-1538 and preferred active a sex steroid precursor DHEA, EM-1304 or EM-01474-D Other compounds of the invention or combination thereof, may be used in place of (or in addition to) EM-800 or EM-1538, DHEA, EM-1304 or EM-01474-D . The concentration of active ingredient may be varied over a wide range as discussed herein. The amounts and types of other ingredients that may be included are well known in the art.

15

Example A

Tablet

| Ingredient | Weight % (by weight of total composition) |
|-------------------|--|
| EM-800 | 5.0 |
| DHEA | 15.0 |
| Gelatin | 5.0 |
| Lactose | 58.5 |
| Starch | 16.5 |

5

Example B

Gelatin capsule

| Ingredient | Weight % (by weight of total composition) |
|----------------------------|--|
| EM-800 | 5.0 |
| DHEA | 15.0 |
| Lactose hydrous | 65.0 |
| Starch | 4.8 |
| Cellulose microcrystalline | 9.8 |
| Magnesium stearate | 0.4 |

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KIT EXAMPLES

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Set forth below, by way of example and not of limitation, are several kits utilizing preferred active SERM EM-800 or EM-1538 and preferred active a sex steroid precursor DHEA, EM-1304 or EM-01474-D. Other compounds of the invention or combination thereof, may be used in place of (or in addition to) EM-800 or EM-1538, DHEA, EM-1304 or EM-01474-D. The concentration of active ingredient may be varied over a wide range as discussed herein. The amounts and types of other ingredients that may be included are well known in the art.

15

Example A

The SERM is orally administered while the sex steroid precursor is percutaneously administered

20

SERM composition
for oral administration (capsules)

| Ingredient | Weight % (by weight of total composition) |
|----------------------------|--|
| EM-800 | 5.0 |
| Lactose hydrous | 80.0 |
| Starch | 4.8 |
| Cellulose microcrystalline | 9.8 |
| Magnesium stearate | 0.4 |

5 Sex steroid precursor composition for topical administration (gel)

| Ingredient | Weight % (by weight of total composition) |
|---|--|
| DHEA | 10.0 |
| Caprylic-capric Triglyceride (Neobee M-5) | 5.0 |
| Hexylene Glycol | 15.0 |
| Transcutol (diethyleneglycol monomethyl ether) | 5.0 |
| Benzyl alcohol | 2.0 |
| Cyclomethicone (Dow corning 345) | 5.0 |
| Ethanol (absolute) | 56.0 |
| Hydroxypropylcellulose (1500 cps) (KLUCEL) | 2.0 |

Example B

10 The SERM and the sex steroid precursor are orally administered

Non-Steroidal Antiestrogen composition for oral administration
(capsules)

| Ingredient | Weight % (by weight of total composition) |
|----------------------------|--|
| EM-800 | 5.0 |
| Lactose hydrous | 80.0 |
| Starch | 4.8 |
| Cellulose microcrystalline | 9.8 |
| Magnesium stearate | 0.4 |

15 Sex steroid precursor composition for oral administration
(Gelatin capsule)

| Ingredient | Weight % (by weight of total composition) |
|----------------------------|--|
| DHEA | 15.0 |
| Lactose hydrous | 70.0 |
| Starch | 4.8 |
| Cellulose microcrystalline | 9.8 |
| Magnesium stearate | 0.4 |

Other SERMs may be substituted for EM-800 or EM-01538 in the above formulations, as well as other sex steroid inhibitors may be substituted for DHEA, EM-1304 or EM-01474-D. More than one SERM or more than one precursor may be included in which case the combined weight percentage is preferably that of the weight percentage for the single precursor or single SERM given in the examples above.

The invention has been described in terms of preferred embodiments and examples, but is not limited thereby. Those of skill in the art will readily recognize the broader applicability and scope of the invention which is limited only by the patent claims herein.